SEPTEMBER 10, 1939

THE JOURNAL OF NUTRITION

VOLUME 18 NUMBER 3

GEORGE R. COWGILL, *Editor*
Yale University School of Medicine, New Haven, Conn.
WILLIAM H. CHAMBERS, *Assistant to the Editor*
Cornell University Medical College, New York City
ASSOCIATE EDITORS

ARTHUR H. SMITH
GRACE MACLEOD
ELMER M. NELSON
L. H. NEWBURGH
JOHN B. BROWN
ALBERT G. HOGAN
JAMES L. GAMBLE
PHILIP A. SHAFFER
ICIE M. HOOBLER
HENRY A. MATTILL
HAROLD H. MITCHELL

Official Organ of the American Institute of Nutrition
PUBLISHED MONTHLY BY
THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY
PHILADELPHIA, PA.
Price, $5.00 per volume, Domestic; $5.50 per volume, Foreign; Two volumes issued annually

Entered as second-class matter January 20, 1934, at the post office at Philadelphia, Pa., under Act of March 3, 1879. Acceptance for mailing at special rate of postage provided for in section 1103, Act of October 3, 1917, authorized on July 2, 1918

SUBSCRIBE

to

NEW ADVANCE ABSTRACT CARD SERVICE

published by

THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY

Planned to meet all the needs of librarians and investigators, this New Advance Abstract Card Service is now offered in the following three styles:

		Annual subscription
Style no. 1.	Advance Abstract Cards in sheets 4 abstracts per sheet—300 mm. by 125 mm.	$2.00
Style no. 2.	Advance Abstract Card Service sheets cut into cards—75 mm. by 125 mm.	2.50
Style no. 3.	Advance Abstract Card Service permanent library card punched— 75 mm. by 125 mm.	3.00, or $5.00 for 2 sets

ADVANTAGES

1. Subject, author, classification and abstract appear on one side. No inverted reading necessary.

2. The Advance Abstract Card in sheets (300 mm. by 125 mm.) can be filed or cut into regular size cards (75 mm. by 125 mm.) for filing. Investigators are thus able to select and keep abstracts of interest only.

3. The service will be issued promptly and months in advance of publication of the manuscript.

4. The service is practical and inexpensive.

5. An index will be furnished to all subscribers annually.

Reproduction of the new form of Advance Abstract Card.

No. 7648

Prouty, William Walter, 1911, and William Henry Cathcart, 1911–
American Institute of Baking

1939. The calcium content of white bread

J. Nutrition, v. 18, no. 3, Sept. 10

Ten control loaves with varying amounts of dry skim milk and dry whole milk were analyzed to determine the effect of milk solids on the calcium content of white bread. As the percentage of milk solids was increased, the percentage of ash and of calcium in the loaf also increased.

Thirty-nine of the most popular commercial loaves of white bread from thirty-three bakeries and twenty-seven states have been analyzed for calcium. The average percentage of calcium found is nearly three times that reported by previous investigators; even the lowest per cent found is more than that previously reported and is equal to that reported by Sullivan for whole wheat berry. The increased use of milk solids and yeast foods has considerably increased the calcium content of commercial white bread.

On the basis of the results, six slices of average commercial white bread will supply approximately 30% of the daily calcium requirement of the average adult, assuming the total amount of calcium is assimilable.

Nutrition — mineral

Issued by The Wistar Institute, July 15, 1939

THE CALCIUM CONTENT OF WHITE BREAD

WILLIAM W. PROUTY AND WILLIAM H. CATHCART
Research Department, American Institute of Baking, Chicago, Illinois

(Received for publication April 21, 1939)

Published analyses of the calcium content of white bread do not give a true picture of the average amount of calcium in modern commercial bread. The data that have been available apparently are the result of investigations made many years ago. During the past few years, however, bakers have been incorporating relatively large quantities of milk solids in white bread, and they often add small amounts of calcium salts to the dough to compensate for deficiencies in local water supplies and to act as yeast nutrients or stimulators and as dough conditioners (hereafter referred to as yeast food). These practices have tended to increase considerably the average calcium content of white bread. In order to ascertain the present quantity of calcium in commercially produced bread, analyses have been made of thirty-nine representative commercial samples and eleven control samples.

Fairbanks ('38) conducted feeding experiments on rats and demonstrated that the addition of milk solids to a water bread (no milk) formula increases the nutritive value of bread. He points out that there is evidence that the nutritive value of bread containing 12% [1] milk solids is of higher order than bread containing 6% milk solids.

Frank and Wang ('25 a) report the calcium content of water bread as 0.029%,[2] Waller ('37) reports 0.027% and Rose

[1] All percentages in this paper referring to ingredients added to bread are on the basis of flour as 100%.

[2] Frank and Wang reported their results in terms of calcium oxide. The above results have been recalculated in terms of per cent calcium.

THE JOURNAL OF NUTRITION, VOL. 18, NO. 3
SEPTEMBER, 1939

('37) reports 0.029%. Rose ('20) gives the calcium content of white bread as 0.032%; Bridges ('35) and Sherman ('37) give 0.031%; and Fiene and Blumenthal ('38) give 0.027%. Neither Rose, Bridges, Sherman, nor Fiene and Blumenthal specify the bread other than as 'white bread.' Frank and Wang ('25 a) report 0.042%[2] calcium for bread containing milk (quantity of milk not given). Rose ('37) reports 0.064% Ca for bread made with milk. Although it was not reported, it is assumed that the results of the above authors are on an 'as received' basis (probably around 34% to 36% moisture).

TABLE 1

Per cent calcium in white bread as reported by various investigators

Per cent calcium 'as received basis' in			
WATER BREAD	BREAD (INGREDIENTS NOT SPECIFIED)	BREAD CONTAINING MILK	INVESTIGATOR(S)
0.029	....	....	Frank and Wang ('25 a)
0.027	....	....	Waller ('37)
0.029	....	....	Rose ('37)
....	0.032	....	Rose ('20)
....	0.031	....	Bridges ('35)
....	0.031	....	Sherman ('37)
....	0.027	....	Fiene and Blumenthal ('38)
....	....	0.042	Frank and Wang ('25 a)
....	....	0.050 (38% moisture)	Sullivan and Howe ('29)
....	....	0.064	Rose ('37)

Sullivan and Howe ('29) report 0.080% calcium (dry basis) for white bread having 5.4% condensed milk and 0.235% yeast food. This would be 0.050% calcium on a 38% moisture basis. These data are summarized in table 1.

Morison and co-workers ('25) gave the following results, per cent calcium (table 2) for experimental loaves, the ingredients being weighed in grams, for varying amounts of dry whole milk. No yeast food was added to these loaves.

It was thought desirable to analyze for calcium a somewhat similar series of commercial control loaves (the ingredients being weighed in pounds and ounces), which con-

tained varying amounts of dry whole milk and dry skim milk and 0.25% yeast food. This was thought advisable in order to approach more closely the average commercial formula.

In addition to this, a request was made to bakeries throughout the country for loaves of their most popular white bread and also the percentage and kind of milk and yeast food, if any, used. The object of this request was to enable the authors to determine the average percentage of calcium in white bread available on the open market in the United States.

TABLE 2

Calcium content expressed in per cent. Analyses made on white bread containing various percentages of dry whole milk on basis of flour as 100% (Morison)

	NO MILK SOLIDS	2.5% MILK SOLIDS	4.0% MILK SOLIDS	5.5% MILK SOLIDS	7.0% MILK SOLIDS	8.0% MILK SOLIDS	9.0% MILK SOLIDS	10.0% MILK SOLIDS
Dry basis	0.016	0.045	0.059	0.069	0.090	0.097	0.111	0.119
38% moisture basis (Calculated by Prouty and Catheart)	0.010	0.028	0.037	0.043	0.056	0.060	0.069	0.074

Loaves have been received and analyzed from six bakeries in Illinois, three bakeries in Louisiana, three bakeries in Tennessee, two bakeries in California, two bakeries in Washington, two bakeries in New York, and one bakery in each of the following states: Nebraska, Minnesota, North Dakota, Florida, Ohio, Maine, Massachusetts, Utah, Colorado, Rhode Island, Missouri, Iowa, Alabama, Kansas, South Carolina, Mississippi, Virginia, Pennsylvania, Montana and Washington, D. C. It is to be understood that these samples were the most popular types of white bread and were not necessarily loaves containing high percentages of milk solids.

EXPERIMENTAL METHOD

Type of ashing dish. A review of the literature failed to reveal the type of container most suited for ashing bread. Consequently, before attempting any work on calcicm analysis,

it was first necessary to determine the type of dish best suited for ashing. Forty separate ash determinations were made in duplicate. Determinations were run in porcelain crucibles and dishes, in silica crucibles and dishes and in platinum crucibles and dishes. The same sample was used throughout. The use of platinum did not meet with favor because it gave a hydrochloric acid solution of the ash a greenish color. The ash also stained the platinum to some extent. With all materials, the per cent ash obtained by the use of shallow dishes was slightly lower than that obtained by the use of crucibles, although both dishes and crucibles gave consistent results. Dishes were not chosen for the work because they were not as convenient to handle as crucibles. Porcelain and silica crucibles checked each other consistently.

Porcelain crucibles (top diameter 53 mm., height 44 mm., capacity 45 cc.) were selected because they were very easy to work with, gave as consistent results as silica, and were also much more economical than silica. On all the above samples, the ashing was done in an electric muffle at 550°C. for 12 hours.

Moisture and ash. The sample was prepared for analysis by the method described in Cereal Laboratory Methods of the A.A.C.C., page 83 ('35). Moisture and ash were run according to the official method of the A.O.A.C., which are described in the Cereal Laboratory Methods, pages 29–30. The vacuum method for moisture was used. For ashing purposes 10 gm. of air dried crumb were used.

Calcium. The analysis for calcium was performed by the Shohl and Pedley ('22) modification of the McCrudden ('09, '10, '11, '12) method. As Shohl ('22 a) pointed out, the hydrogen-ion-concentration is an important factor in making a calcium determination. If the solution is more acid than pH 4.0, calcium oxalate is dissolved, and if it is less acid than pH 5.6, magnesium ammonium phosphate is precipitated. Shohl and Pedley's modification consisted in precipitation at a specific pH. The only modifications on Shohl and Pedley's method were to leave out digesting with ammonium persulfate,

which was not necessary in bread analysis, and to titrate with 0.02N $KMno_4$ instead of 0.05N $KMno_4$.

Blanks were run on all reagents used along with every determination. Although improbable, tests were run to determine if any calcium was dissolved from the crucible with the hydrochloric acid used for dissolving the ash. The tests were negative in every case.

RESULTS AND DISCUSSION

Using the facilities of the Institute, several of the students were engaged in baking the control loaves used in the first part of the analysis. Ten pounds of flour per batch for each dough and 0.25% yeast food were used. Doughs were made containing no milk, 2, 4, 6, 8, and 10% dry skim milk and another series containing 2, 4, 6, 8, and 10% dry whole milk. The results are reported in table 3. Calculations of results to dry basis and 38% moisture basis were made separately from results of analysis on air dry crumb.

The results in table 3, as was to be expected, show that the per cent ash and per cent calcium increase as the per cent milk increases. Dry skim milk when used on the same percentage basis as dry whole milk gives higher calcium values. It will be noted that in general with each kind of milk the per cent calcium increases in fairly uniform increments with the exception of samples 11 and 13 for dry whole milk. The only explanation of this discrepancy is that the bread samples were made on a small commercial scale and weighed in pounds and ounces. It is evident that large errors could be introduced by a slight inaccuracy of weighing in pounds and ounces a batch containing 10 pounds of flour.[3]

Although most of the results for dry whole milk are in fairly good agreement with those of Morison and co-workers, there are discrepancies. About the only explanation that can be offered is that Morison's breads were made with ingredients which perhaps had slightly different calcium con-

[3] All samples which did not fall in line were re-run in duplicate, but in all cases the results checked within 0.003% calcium.

TABLE 3

Per cent ash and calcium of control loaves made with various percentages of dry skim milk (D.S.M.) and dry whole milk (D.W.M.) on the basis of flour as 100%; 0.25% Arkady (yeast food) was used in each dough

SAMPLE NO.	PER CENT MILK BASED ON FLOUR AS 100%	TYPE OF MILK	PER CENT WATER IN ORIGINAL LOAF[1]	PER CENT WATER IN AIR DRY CRUMB	PER CENT ASH 38% MOISTURE		PER CENT ASH DRY BASIS		PER CENT CALCIUM 38% MOISTURE		PER CENT CALCIUM DRY BASIS	
					Duplicate	Average	Duplicate	Average	Duplicate	Average	Duplicate	Average
					1.75		2.81		0.025		0.040	
5	0	....	30.0	8.2	1.75	1.75	2.81	2.81	0.025	0.025	0.040	0.040
					2.06		3.32		0.038		0.062	
6	2	DSM	29.0	8.0	2.07	2.07	3.34	3.33	0.040	0.039	0.065	0.064
					2.07		3.34		0.051		0.083	
7	4	DSM	29.7	8.0	2.07	2.07	3.34	3.34	0.052	0.052	0.084	0.084
					2.14		3.45		0.062		0.100	
8	6	DSM	29.4	8.3	2.14	2.14	3.46	3.46	0.063	0.063	0.102	0.101
					2.29		3.71		0.080		0.130	
9	8	DSM	29.7	8.2	2.29	2.29	3.71	3.71	0.081	0.081	0.130	0.130
					2.31		3.74		0.099		0.160	
10	10	DSM	29.4	7.9	2.30	2.31	3.72	3.73	0.096	0.097	0.156	0.158
					2.20		3.56		0.040		0.065	
11	2	DWM	28.4	7.5	2.20	2.20	3.56	3.56	0.041	0.041	0.067	0.066
					2.10		3.39		0.041		0.067	
12	4	DWM	27.7	7.5	2.10	2.10	3.39	3.39	0.043	0.042	0.070	0.069
					2.64		4.25		0.046		0.074	
13	6	DWM	28.5	7.4	2.64	2.64	4.25	4.25	0.047	0.047	0.077	0.076
					2.21		3.57		0.060		0.097	
14	8	DWM	27.8	7.4	2.21	2.21	3.57	3.57	0.060	0.060	0.097	0.097
					2.47		3.98		0.074		0.121	
15	10	DWM	28.1	7.3	2.45	2.46	3.96	3.97	0.073	0.074	0.119	0.120

[1] Loaves were not wrapped and moisture was not determined until 24 hours after baking.

tents than those used by the authors and contained no yeast food. This undoubtedly accounts for the lower calcium content of Morison's water bread. Thus, any increase in the milk content of bread would have a greater effect on a corresponding increase in per cent calcium of bread made from Morison's formula than bread made from the formula used by the authors.

The results for water bread of Frank and Wang ('25 a), Rose ('37) and Waller ('37) agree with the result of 0.025% calcium on 38% moisture basis as reported in table 3. The per cent calcium reported by Rose ('20), Bridges ('35), Sherman ('37), and Fiene and Blumenthal ('38) for 'white bread' are only slightly higher.

The results of the commercial breads which have been analyzed are reported in table 4.

From table 4 the average per cent calcium for the thirty-nine loaves, representing twenty-seven states, was 0.128 on dry basis and 0.080 on 38% moisture basis. The lowest figure found was 0.048% on a 38% moisture basis and 0.076% calcium on dry basis. The highest figure was 0.145% on a 38% moisture basis and 0.235% calcium on dry basis. The average ash was 1.86% on a 38% moisture basis and 3.00% on a dry basis. It is interesting to point out that the average per cent calcium found for white bread (38% moisture basis) is much higher than 0.048% calcium (dry basis) for wheat, as reported by Sullivan ('33). In the same article Sullivan reports 0.016% calcium (dry basis) for patent flour which gives some idea of the large increase in calcium of average white bread over the patent flour from which it is made.

Due to the increased amount of milk being used today in the bake shop, and also to the use of yeast foods, the calcium content of commercial white bread on the market today is much higher than previously reported. In white bread today, the percentage of milk is not necessarily an indication of the percentage of calcium; in some cases the calcium content is higher than would be indicated from milk content alone because of the various types and percentages of yeast foods being used

and because some yeast foods contain relatively large quantities of calcium salts.

Sherman ('37) points out that the human adult needs on the average a minimum of 0.45 gm. of calcium per day. Assuming that an average person consumes six slices of average white

TABLE 4

Per cent ash and per cent calcium of commercial white bread as sold on the open market in the United States

NUMBER OF BRANDS ANALYZED	PER CENT MILK BASED ON FLOUR AS 100%	TYPE OF MILK	AVERAGE PER CENT MOISTURE IN LOAF 'AS RECEIVED'	AVERAGE PER CENT ASH 38% H_2O BASIS	AVERAGE PER CENT ASH DRY BASIS	AVERAGE PER CENT CALCIUM 38% H_2O BASIS	AVERAGE PER CENT CALCIUM DRY BASIS
1	2.0	D.S.M.	35.4	1.84	2.96	0.065	0.104
2	3.0	D.S.M.	34.1	1.83	2.96	0.066	0.106
1	3.5	D.S.M.	34.3	1.83	2.96	0.072	0.114
3	4.0	D.S.M.	34.5	1.88	3.03	0.070	0.112
4	5.0	D.S.M.	33.3	1.81	2.92	0.076	0.122
4	6.0	D.S.M.	34.9	1.83	2.95	0.090	0.145
2	10.0	D.S.M.	34.5	2.03	3.28	0.141	0.229
1	7.0	D.W.M.	31.4	2.01	3.24	0.083	0.134
1	4.25	S.C.M.	35.1	1.76	2.84	0.055	0.089
1	15.0	S.C.M.	34.9	1.96	3.16	0.055	0.089
1	8.0	C.M.	34.5	1.69	2.72	0.051	0.082
1	9.0	C.M.	33.0	1.76	2.84	0.070	0.112
1	11.7	C.M.	34.8	1.87	3.10	0.059	0.095
1	10.0	S.C.S.M.	35.0	1.78	2.87	0.053	0.085
1	13.4	S.C.S.M.	24.1	1.93	3.12	0.118	0.191
10	Not reported	....	34.2	1.88	3.03	0.075	0.120
3	5.0	P.M.	34.1	1.83	2.95	0.087	0.142
1	{ 4.0 { 5.0	P.M. D.S.M.	34.0	1.96	3.16	0.114	0.184

DSM represents dry skim milk; DWM, dry whole milk; SCM, sweetened condensed milk; CM, condensed milk; SCSM, sweetened condensed skim milk; and PM, powdered milk (further details on this sample were not obtained from the baker).

bread per day, i.e., two slices at each meal, and that each slice weighs 1 ounce (28.35 gm.), which it does on the average, then, on the basis of the results obtained in this paper, this person will receive 0.136 gm. of calcium per day from bread.

This is 30% of his total calcium requirement for that day, assuming, of course, that all this calcium is assimilable.

Sherman ('37) and Fiene and Blumenthal ('38) report liquid whole milk as containing 0.118% to 0.120% calcium. Assuming one glass of milk is ½ pint, it will then contain on an average 0.285 gm. of calcium. Variable amounts of calcium may be obtained in vegetables and other foods. However, one glass of milk and six slices of bread a day (two slices at each meal) will furnish approximately an adult's total daily calcium requirement.

SUMMARY

Ten control loaves with varying amounts of dry skim milk and dry whole milk were analyzed to determine the effect of milk solids on the calcium content of white bread. As the percentage of milk solids was increased, the percentage of ash and of calcium in the loaf also increased.

Thirty-nine of the most popular commercial loaves of white bread from thirty-three bakeries and twenty-seven states have been analyzed for calcium. The average percentage of calcium found is nearly three times that reported by previous investigators; even the lowest per cent found is more than that previously reported and is greater than that reported by Sullivan for whole wheat berry. The increased use of milk solids and yeast foods has considerably increased the calcium content of commercial white bread.

On the basis of the results, six slices of average commercial white bread will supply approximately 30% of the daily calcicm requirement of the average adult, assuming the total amount of calcium is assimilable.

LITERATURE CITED

BRIDGES, M. A. 1935 Food and beverage analyses. Lea and Febiger, Philadelphia, Pa.

FAIRBANKS, B. W. 1938 Improving the nutritive value of bread by the addition of dry milk solids. Cereal Chem., vol. 15, pp. 169–179.

FIENE, F., AND S. BLUMENTHAL 1938 Hand book of food manufacturing. Chem. Pub. Co., New York, N. Y.

FRANK, M., AND CHI CHE WANG 1925 Variation in the calcium content of common foods. J. Home Economics, vol. 17, pp. 494–497.

MCCRUDDEN, F. H. 1909–1910 The quantitative separation of calcium and magnesium in the presence of phosphates and small amounts of iron devised especially for the analysis of foods, urine and feces. J. Biol. Chem., vol. 7, p. 83.

———— 1911–1912 Ibid., vol 9, p. 187.

MORISON, C. B., AND CO-WORKERS 1925 Composition of milk bread. Baking Technology, vol. 4, pp. 48–50.

ROSE, M. S. 1920 Experiments on the utilization of carrots by man. J. Biol. Chem., vol. 41, pp. 349–355.

———— 1937 A laboratory handbook for dietetics. Macmillan and Co., New York, N. Y.

SHERMAN, H. C. 1937 The chemistry of food and nutrition. Macmillan and Co.

SHOHL, A. T. 1922 The effect of hydrogen-ion-concentration upon the determination of calcium. J. Biol. Chem., vol. 50, pp. 527–536.

SHOHL, A. T., AND F. G. PEDLEY 1922 A rapid and accurate method for calcium in urine. J. Biol. Chem., vol. 50, pp. 537–544.

SULLIVAN, B. 1933 The inorganic constituents of wheat and flour. Cereal Chem., vol. 10, pp. 503–514.

SULLIVAN, B., AND M. HOWE 1929 Minerals of wheat. I. Sulfur and chlorine. Cereal Chem., vol. 6, pp. 396–400.

WALLER, D. S. 1937 Nutritive value of foods. Geo. Wahr, Ann Arbor, Mich.

SOME HISTOPATHOLOGIC OBSERVATIONS ON CHICKS DEFICIENT IN THE CHICK ANTI-DERMATITIS FACTOR OR PANTOTHENIC ACID [1]

PAUL H. PHILLIPS AND R. W. ENGEL

Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison [2]

FOUR FIGURES

(Received for publication April 27, 1939)

It has been frequently observed that chicks on a ration low in the chick antidermatitis factor or pantothenic acid, developed neuromalacia. Generally the paralysis did not reach the typical 'curled toe' stage, but a slight paralysis was apparent. This paralysis was largely prevented by the administration of crystalline riboflavin.

Previous work ('38) has shown that riboflavin prevented neuromalacia of the peripheral nerves and that it would not completely prevent neuropathology in the spinal cord. Preliminary observations indicated that antidermatitis factor concentrates largely prevented the structural changes of the cord in chick dermatitis. It seemed desirable to determine whether one or both of these factors were involved in the nervous symptoms found in chick dermatitis. To this end a number of chicks suffering from dermatitis were examined from time to time. It soon became evident that systematic studies of this disease were necessary. With this in mind a general survey of the histopathology has been made and is herewith reported.

[1] Published with the approval of the director of the Wisconsin Agricultural Experiment Station.

[2] These studies were aided in part by grants from the Research Funds of the University and from the Works Progress Administration.

EXPERIMENTAL

All birds studied in these experiments were day-old White Leghorn chicks fed diet 241H. This diet is a heated diet used by Mickelsen and his associates ('38). It produces marked dermatitis and death in the growing chick if unsupplemented by the antidermatitis factor. The first experiment[3] consisted of five lots of birds as follows:

Lot I—Basal ration 241H only.
Lot II—Basal ration + antidermatitis factor concentrate = 40% liver.
Lot III—Basal ration + riboflavin (4 μg. per gram of ration).
Lot IV—Basal ration + riboflavin (same as above) + B_1 + nicotinic acid.
Lot V—Basal ration + antidermatitis factor (same as in I) + riboflavin (same as in III).
Lot VI—Basal ration + 10 parts blackstrap molasses.

From five to ten birds were microscopically examined from each of these lots. A second experiment was likewise available. This experiment was composed of five lots of four birds each. One was fed 241H only, two lots were fed 241H plus 1% and 2% liver respectively for positive control and the remaining lots were fed the synthetic peptide made by coupling the acid chloride fraction of the alkali inactivated liver concentrate with the β-alanine ester as described by Woolley et al. ('39). Some of the birds reported by them were used for histologic study by us.

The sciatic nerve, spinal cord, liver and kidney were routinely examined with a few observations on the thymus, thyroid, striated muscle and skin.

The usual histologic technics were employed throughout these studies. The sciatic nerves were studied by means of the polarizing microscope, the Marchi reaction and by silver impregnation of axis cylinders. The spinal cords were studied for Nissl material by Einarson's method ('32), by the Marchi reaction and by the method of Bodian ('36). All other tissues were studied with the aid of hematoxylin and eosin.

[3] We are indebted to Mr. Harry A. Waisman for his cooperation in rearing the birds used in these experiments.

RESULTS

The observations made in these studies are summarized in table 1. It is seen that the spinal cord structures were affected in 100% of the cases unless concentrates of pantothenic acid were supplied. If the pantothenic acid concentrate was given without riboflavin neuromalacia developed in all cases. When the procedure was reversed and riboflavin

TABLE 1

This shows the number of chicks which developed histopathologic changes in the various organs and tissues that were studied

RATION FED	NUMBER OF CASES	NERVOUS SYSTEM		OTHER TISSUES				
		Sciatic	Spinal cord	Liver	Thymus	Skin	Muscle	Thyroid
241H only	14	5	14	1	2	12	1	..
241H + a.f.conc.[1]	10	10	0	..	1	0	1	0
241H + riboflavin	8	0	8	4	2	8	0	1
241H + a.f.conc. + riboflavin	5	0	0	2	0	0	0	0
241H + B_1 + riboflavin + nicotinic acid	7	0	7	0	2	..	..	..
241H + 10% molasses	3	0	0	0	..	..	..	..
241H + synthesis	6	3	0	0	..	..	..	..
241H + 1% liver	2	0	1	..	0	0	..	..
241H + 2% liver	4	4	1	0	0	0	0	0

[1] Antidermatitis factor concentrate.

alone was added, no changes in the sciatic nerve were observed. Here, however, degeneration in the spinal cord was present in all cases. If both a pantothenic acid concentrate and riboflavin were added, no changes were evident in either the spinal cord or the sciatic nerve. Nicotinic acid was ineffective in preventing the cord changes. The feeding of the pantothenic acid concentrate, made by the coupling of the acid chloride part of the alkali-inactivated liver concentrate with β-alanine ester, was effective in preventing cord changes, but the chicks developed histologic signs of neuromalacia. Liver at 1 and 2% was partially effective but was somewhat variable.

In most cases other tissues were less regularly affected. Occasionally the livers showed hydropic and fatty degeneration. If extra amounts of thiamin were added to the diet riboflavin was not effective in preventing these fatty livers. In not a few cases the thymus was in an advanced state of involution. This seemed to occur in those birds lacking in either pantothenic acid or riboflavin. It did not occur when both were present.

All cases of sciatic neuropathology were typical neuromalacia cases with myelin swelling, degeneration, clubbing and fragmentation of axis cylinders and Schwam cell proliferation as previously described ('38).

Lesions in the spinal cord were characterized by the myelin degeneration of myelinated fibers distributed widely throughout the white matter, except in the posterior region (figs. 1 to 4). Such degenerating fibers were observed to occur in all segments of the cord down to the lumbar region. In the brachial segment nerve cells show chromatolysis of the Nissl material. Such cells often presented an abnormal distribution of the Nissl which seemed to be an early stage of the more complete loss of the Nissl. These cases did not show a pronounced gliosis. It was noticed that in silver preparations vacuolar spaces were often observed in the cord cross section. In longitudinal sections degenerative changes were frequently observed in the axis cylinders of the affected nerves. In severe cases the nerve cells show fibrillae fragmentation.

Observations on the skin showed a dry sloughing of the keratinized epithelium. However, incrustations about the corners of the mouth and the areas between the toes usually showed distinct characteristics of dermatitis.

These studies support the preliminary observations published earlier with respect to the effect of the antidermatitis factor upon the neuropathology of the spinal cord ('38). It appears that pantothenic acid is definitely established as a constituent of and necessary to the maintenance of the normal intact elements of spinal cord.

It is seen that 10% molasses (blackstrap) protected against cord lesions and is indicative of its pantothenic acid content. Many times we have experienced increased severity of neuromalacia pathology when a ration low in riboflavin and pantothenic acid was supplemented by molasses. In these studies however there were no signs of sciatic pathology.

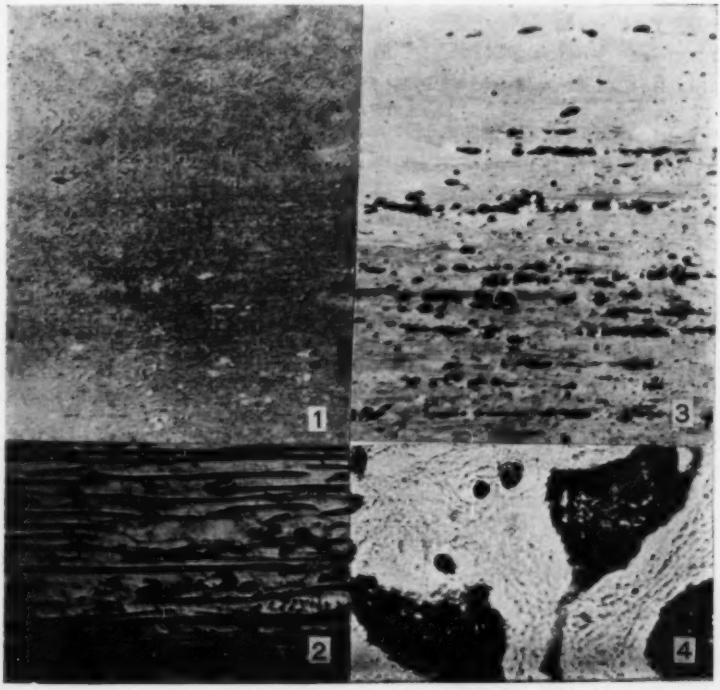


Fig. 1 Marchi preparation of the spinal cord of a chick fed ration 241H + pantothenic acid concentrate + riboflavin. × 400.

Fig. 2 Marchi section of the spinal cord of a chick fed ration 241H + thiamin + nicotinic acid + riboflavin. × 400.

Fig. 3 Bodian silver preparation of the spinal cord of a chick fed ration 241H only. Note the fragmentation of axis cylinders near the center of the field. × 400.

Fig. 4 Gallocyanin preparation from a chick fed ration 241H only. Chromatolysis and early Nissl changes probably due to lack of riboflavin. × 900.

No pathologic changes were observed in the brains of these chicks although the degenerating fibers high in the spinal cord would lead one to suspect some neuropathology in the higher centers. In these studies we have followed only the gross histopathologic aspects. The more minute histologic detail must await further study.

These studies show that the addition of pantothenic acid and riboflavin are necessary to eliminate neuropathology in chicks fed ration 241H. The former is necessary for the intact spinal cord and the latter for the peripheral nerves.

SUMMARY AND CONCLUSIONS

These data indicate that pantothenic acid is necessary for the maintenance of the normal intact structures of the spinal cord. Nicotinic acid, riboflavin or vitamin B_1 were not effective in preventing the spinal cord pathology in chicks fed ration 241H, while molasses proved to be effective. Chicks suffering from dermatitis often showed thymus involution, liver damage and keratitis and dermatitis in the skin. Fatty livers frequently occurred and were not prevented by riboflavin when thiamin was also fed. The synthetic pantothenic acid made from the β-alanine ester and the acid chloride portion of the alkali-inactivated liver concentrate were highly effective in eliminating the spinal cord pathology.

Pantothenic acid is necessary for the prevention of the neuropathology of the spinal cord in chicks suffering from dermatitis.

LITERATURE CITED

BODIAN, D. 1936 A new method for staining nerve fibers and nerve endings in mounted paraffin. Section 1, Anat. Rec., vol. 36, p. 89.

EINARSON, L. 1922 A method for progressive selective staining of Nissl and nuclear substances in nerve cells. Am. J. Path., vol. 8, p. 295.

MICKELSEN, O., H. A. WAISMAN AND C. A. ELVEHJEM 1938 The inactivity of nicotinic acid in chick dermatitis. J. Biol. Chem., vol. 124, p. 313.

PHILLIPS, P. H., AND R. W. ENGEL 1938 The histopathology of neuromalacia and 'curled toe' paralysis in the chick fed low riboflavin diets. J. Nutrition, vol. 16, p. 451.

WOOLLEY, D. W., H. A. WAISMAN AND C. A. ELVEHJEM 1939 Nature and partial synthesis of the chick antidermatitis factor. J. Am. Chem. Soc., vol. 61, p. 977.

OXALIC ACID IN FOODS AND ITS BEHAVIOR AND FATE IN THE DIET

E. F. KOHMAN[1]

Campbell Soup Company, Camden, N. J.

THREE FIGURES

(Received for publication April 25, 1939)

One is apt to gather from nutritional literature that vegetables do not supply calcium efficiently. Because this is not in accord with the results of a number of experiments, some covering several generations of animals (Kohman, Eddy and associates, '31, '34), this discrepancy presented itself as a problem wanting solution. As there seemed to be confusion about the amount of oxalates in foods and a scarcity of data as to the behavior and fate of oxalates in the diet, information on these points might be expected to throw light on the availability of calcium. It seemed desirable for higher accuracy, to make oxalate determinations under conditions that would avoid drastic treatment of the food and entail the concentration of the oxalate of a relatively large sample, freed from most of the other food constituents.

METHOD OF ANALYSIS

The following procedure was used. The samples, 400 gm., were first cooked tender by steaming without drainage loss. After thorough disintegration and an approximation of water content, sufficient concentrated hydrochloric acid was added to yield a 15% solution in the water present. This was

[1] The experimental work embodied in this paper was done in 1934–1936 while the author was with the Research Laboratory of the National Canner's Association, Washington, D. C. There he had the assistance of N. H. Sanborn and D. C. Smith. This opportunity is taken to acknowledge their part in the work.

allowed to stand at room temperature, with occasional stirring, not less than 2 days.

The sample was then pressed in cheese cloth and the weights of the drained material and of the residue recorded. By direct titration of a highly diluted sample, after filtration if necessary, the chlorine in the two fractions was determined. On the assumption that the oxalic acid distributed itself in the same ratio as the chlorine, it could be calculated for the entire sample by a determination on the drained fraction. This assumption was proved correct for pineapples and turnip greens by adding varying amounts of oxalic acid and subsequent analysis.

A 250 to 300 cc. sample of the drained liquid was subjected to 24 hours of wet ether extraction in an apparatus modeled after that suggested by Palkin, Murray and Watkins ('25) and suitably proportioned for this purpose using sintered glass to disperse the ether. In this the liquid to be extracted was 32 to 37 cm. deep and the ether coursed up through it from the 2.6 sq.cm. of fritted glass at a rate of 20 cc. per minute. When the extraction was complete, water was added to the extract, the ether evaporated and the water solution filtered. Calcium oxalate was then precipitated. After washing and redissolving a second, and if necessary, a third precipitation was made for complete purification. The precipitate was then titrated with permanganate. By precipitating the calcium in the titrated medium as calcium oxalate, the purity of the original oxalate could be gauged. All determinations were made in duplicate and the average is given in table 1. To study varietal and other factors more than one sample of some products were analyzed. As no significance could be placed on the differences, all the results were averaged and the number of samples is given in parenthesis.

THE OCCURRENCE OF OXALATES IN FOODS

Only a few foods, notably spinach, Swiss chard, New Zealand spinach, beet tops, lamb's quarter, poke, purslane and rhubarb have a high oxalate content. In them, expressed

TABLE 1

Oxalic acid (anhydrous) in foods

Product	*Total solids*	*Oxalic acid*	*Calcium*
Vegetables	%	%	%
Asparagus	6.05	0.0052	0.0201
Beans, green pod (3)	9.58	0.0310	0.0440
Beans, wax	7.80	0.041	0.054
Beans, lima (2)	24.25	0.0043	0.044
Beets, unpeeled	8.23	0.138	0.018
Beet leaves	6.60	0.916	0.120
Beet stems	6.66	0.338	0.040
Broccoli, leaves and flowers	10.50	0.0054	0.21
Broccoli, stalks	7.57	0.0035	0.092
Cabbage (2)	8.80	0.0077	0.189
Cabbage sprouts	8.52	0.0059	0.150
Cabbage, Chinese	6.45	0.0073	0.210
Carrots	11.02	0.033	0.044
Cauliflower	8.90	None	0.034
Celery stalks, bleached	4.58	0.034	0.054
Celery, soup leaves	14.66	0.050	0.55
Celery, soup stems	10.20	0.062	0.18
Collards (2)	12.75	0.0091	0.361
Chard, Swiss, leaves	9.47	0.66	0.11
Chard, Swiss, stalks	7.10	0.29	0.045
Chard, Swiss, leaves and stalks (2)	8.28	0.645	0.129
Chenepodium (Lamb's quarter)	8.20	1.11	0.099
Corn, sweet, white	25.00	0.0014	0.0076
Corn, sweet, yellow (2)	33.51	0.0052	0.0033
Cress, land, wild	15.00	None	0.24
Cress, early fine curled	8.80	0.0106	0.182
Cucumbers	3.72	None	0.014
Dandelions (3)	11.38	0.0246	0.171
Egg plant	6.18	0.0069	0.010
Endive (5)	7.58	0.0273	0.105
Escarole	6.10	0.0116	0.087
Kale	11.05	0.013	0.31
Kale, minus leaf ribs	18.05	0.011	0.294
Lettuce (6)	6.46	0.0071	0.073
Mustard greens (3)	8.40	0.0077	0.235
Okra	13.20	0.048	0.077
Onions, green	13.65	0.023	0.057
Parsley	13.70	0.19	0.29
Parsnips	22.70	0.010	0.049
Peas	19.50	None	0.019
Peppers, sweet, green (3)	7.34	0.016	0.0135
Poke	7.74	0.476	0.052
Potatoes, Irish	20.38	0.0057	0.0094
Potatoes, sweet	33.60	0.056	0.034
Purslane, leaves	9.45	0.910	0.13
Purslane, stalks	8.44	0.518	0.067
Radishes	3.75	None	0.028

TABLE 1—*Continued*

Product	*Total solids*	*Oxalic acid*	*Calcium*
Vegetables	%	%	%
Rape	10.82	0.0015	0.11
Rhubarb	6.62	0.50	0.044
Spinach (53)	10.35	0.892	0.122
Spinach, canned (12)	7.15	0.364	0.058
Spinach, New Zealand, leaves	7.60	0.89	0.11
Spinach, New Zealand, stalks	8.26	0.65	0.083
Squash, green summer	5.51	None	0.036
Turnips, peeled	8.16	None	0.037
Turnips, unpeeled	6.58	0.0018	0.028
Turnip greens	8.25	0.0146	0.245
Fruits			
Apples, early summer	12.58	None	0.010
Apricots	13.62	0.014	0.024
Avocados	14.60	None	0.0095
Bananas	23.81	0.0064	0.0071
Berries, black	12.25	0.018	0.038
Berries, blue	20.81	0.015	0.026
Berries, black rasp	22.10	0.053	0.058
Berries, dew	13.70	0.014	0.027
Berries, green goose	13.05	0.088	0.023
Berries, red rasp	14.10	0.015	0.023
Berries, straw	10.48	0.019	0.031
Cherries, red sour	12.18	0.0011	0.010
Cherries, sweet, Bing	24.50	None	0.0019
Currants, red	15.52	0.019	0.030
Grapes, Concord	15.30	0.025	0.024
Grapes, Thompson's seedless	23.90	None	0.013
Grapefruit	11.50	None	0.015
Lemons, juice	9.20	None	0.011
Lemons, peel	18.90	0.083	0.17
Limes, juice	10.39	None	0.015
Limes, peel	31.00	0.11	0.26
Mangoes	15.35	None	0.015
Melons, cantaloup	8.46	None	0.0090
Melons, casaba	11.22	None	0.0054
Melons, honey dew	6.08	None	0.0090
Melons, water	10.42	None	0.0060
Nectarines	14.45	None	0.0084
Oranges, edible portion	15.15	0.024	0.038
Oranges, peel	22.90	0.078	0.15
Peaches, Alberta	15.68	0.0050	0.012
Peaches, Hiley	14.10	None	0.0089
Pears, Bartlett	17.60	0.0030	0.014
Pineapples, Hawaiian canned	17.44	0.0063	0.019
Plums, damson	11.70	0.010	0.015
Plums, green gage	13.20	None	0.0080
Prunes, Italian	15.76	0.0058	0.12
Tomatoes	5.76	0.0075	0.010

as anhydrous oxalic acid, it is often considerably over 10% on a dry basis. One per cent in table 1 is equivalent to 1.4% of the usual crystalline form of oxalic acid. In most foods there are present mere traces. It is notable, however, that oxalate was obtained from all but a very few products, mostly fruits.

One purpose of the analytical survey was to ascertain if some varieties of spinach might be relatively free of oxalate. This hope was not realized. In fifty-three samples, including practically all commercial and many experimental varieties grown in California and in Maryland as well as those shipped from Texas, Florida and Carolina, the average anhydrous oxalic acid content was 9.02% on the dry basis (maximum 12.6, minimum 4.5). The calcium values averaged 1.25% (maximum 2.50, minimum 0.44). California spinach was only slightly lower in oxalic acid but markedly lower in calcium. California spinach averaged 0.59% calcium (maximum 0.84, minimum 0.44) while Maryland spinach averaged 1.92% (maximum 2.50, minimum 1.42). Since a considerable number of mineral elements are dietary essentials, such a variation raises a number of important questions.

PLAN OF FEEDING EXPERIMENTS

As a number of widely used greens contain insignificant amounts of oxalates, a comparison of these with spinach seemed in order. Green leaf-vegetables are our richest calcium sources. In previous experiments Kohman, Eddy and White ('37) used diets of canned foods and found them to supply calcium efficiently. For convenience, therefore, a basal diet of one can each of roast beef, peas, carrots and sweet potatoes was chosen. The essential analysis expressed in grams was:

One can	*Net weight*	*Solids*	*Calcium*	*Phosphorus*
Roast beef	362	133	0.054	0.155
Peas	607	111	0.106	0.474
Carrots	599	45	0.147	0.156
Sweet potatoes	551	184	0.133	0.251

By securing a sufficient amount from one lot of each of these, it was possible to have a constant, uniform supply for

an entire series of experiments conducted over an extended period of time. These four foods, in the above proportion, were thoroughly mashed and mixed, liquid and all, and hence had to be eaten in that proportion. This diet was obviously low in calcium, i.e., 0.093%. It permitted good but not maximum growth and bone formation, evincing efficient calcium. Small additions of greens (5 to 8%) were made to supply 60% of the calcium of the final mixture. This raised the calcium content to 0.22%.

In experiment 3, table 2, some additions to the basal diet were on a different basis. The turnip greens in diet C supplied only 58.8% of the calcium. It was added in that amount to be compared with diet D, in which it was accompanied by an equal amount of spinach, which was half the amount of spinach added in diet B to supply 60% of the calcium. The two greens in diet D supplied 70% of the calcium and raised the content to 0.3%. Also in experiment 3, diet D was planned to be equal to diets B and E on the basis of crude fiber, since the effect of crude fiber on calcium availability has often been questioned. Diet E is equal to D both in fiber and calcium.

The rats used were purchased from a large dealer who was able to supply large litters suitable for apportioning between the diets of a given experiment equally as to weight and sex. They were started on the experiments at 21 or 22 days of age when they weighed 30 to 35 gm. Six animals were always placed on each diet and kept in one cage with a raised wire bottom, three mesh to the inch. Except as noted otherwise the duration of the experiments was 21 days. There was an occasional death during this period and in every case it was an animal receiving either spinach or calcium oxalate.

RESULTS OF FEEDING EXPERIMENTS

It is apparent from data in tables 2, 3 and 4 that whereas spinach greatly increases the calcium content of the low calcium but well performing basal diet, it decidedly interferes with both growth and bone formation. This cannot be ex-

TABLE 2

Growth of rats and bone ash of alcohol-ether extracted dry tibia

Dietary addition		*Weight gain*	*Ash in tibia*
Experiment 1—21 days			
		gm.	*%*
Turnip greens (4.5%)		59	43.2
Spinach (8.2%)		38	33.4
Experiment 2—28 days			
None		67	44.7
Calcium carbonate		94	50.0
Calcium oxalate		79	47.6
Turnip greens (4.5%)		106	51.6
Spinach (8.2%)		77	45.2
Experiment 3—21 days			
None	Diet A	54	44.1
Spinach (8.8%–23.24 gm.)	Diet B	40	37.8
Turnip greens (4.4%–11.62 gm.)	Diet C	87	53.1
Turnip greens 11.62 gm. + spinach 11.62 gm.	Diet D	74	47.9
Spinach 23.24 gm. + $CaCO_3$ 0.406 gm.	Diet E	46	39.5
Experiment 4—21 days			
None		53	45.3
Canned spinach brand 1		47	40.3
Canned spinach brand 2		58	40.9
Canned spinach brand 3		57	43.5
Freshly cooked spinach		62	41.1
Experiment 6—21 days			
Calcium carbonate		76	51.1
Spinach, + $CaCO_3$ equivalent to oxalate in spinach		88	53.4
Spinach (7%)		57	40.5
Kale (6%)		79	53.5
Mustard greens (5%)		84	53.2
Experiment 7—21 days			
None		52	47.3
Canned spinach brand 1—not drained		45	45.7
Canned spinach brand 1—drained		54	45.1
Canned spinach brand 3—not drained		52	44.8
Canned spinach brand 3—drained		56	46.0
Freshly cooked spinach—not drained (9%)		54	45.5
Freshly cooked spinach—drained (7%)		75	49.1

plained on the basis of crude fiber. On the other hand greens with negligible oxalate content, such as turnip greens, kale, mustard greens and collards, markedly improve growth and bone formation under similar conditions. If enough calcium carbonate is added with the spinach to balance stoichiometrically its oxalate, performance in the rat is then comparable with other greens. This would require, in the fifty-three samples analyzed, from two to nine times as much calcium as the spinach contains. A general impression was obtained that some superiority, such as sleekness of fur, resulted when a low oxalate bearing green supplied calcium as against an equivalent addition of calcium carbonate.

In table 2 are recorded the results of a series of experiments in which the gain in weight and the per cent ash in the tibia was determined after feeding the basal diet alone and also the basal diet with various additions, to similar groups of animals. Each figure represents the average of six animals.

Table 3 records the gain in weight and the gain in calcium per animal in other similar feeding experiments. To arrive at the calcium per animal at the time the feeding was started, a similar group of six animals was sacrificed and their calcium content determined. In all cases when an entire animal was analyzed, the food was removed the evening before, i.e. 16 hours. In experiment 8, table 3, the food consumption was determined and from this the calcium utilization obtained. It appears that when turnip greens were added to the basal diet 79% of the calcium was utilized but when spinach was added only 15% was utilized. Assuming that the calcium supplied by the basal diet and the turnip greens was equally available, and bearing in mind that 15% of the total calcium is only 38% of that supplied by the basal diet, it appears that the spinach not only supplied no available calcium, but it actually rendered unavailable 41% of the calcium of the basal diet, i.e., the difference between 79 and 38. The sample of spinach used had 10.1% oxalic acid and 1.53% calcium. The diet to which it was added then had 0.8% oxalic acid. When calcium oxalate was the addition, 44% of the calcium was

utilized, showing that the rat can make use of a small portion of the calcium in it. It is notable that with turnip greens in the diet four times as much calcium per gram of tissue was deposited as with spinach in the diet. The data in tables 3 and 4, experiment 8, indicate a superiority of turnip greens over calcium carbonate in growth, calcium utilization and calcium deposition per unit body weight.

TABLE 3

Growth record and gain in calcium

Addition to basal diet	*Gain in weight*	*Calcium per rat*		*Calcium gain per gram gain in weight*	*Calcium utilized*
		Total	Net gain		
Experiment 5—21 days					
	gm.	*gm.*	*gm.*	*gm.*	*%*
Controls—21 days old	—	0.1853	—	—	—
Calcium oxalate	62	0.3535	0.1682	0.00267	—
Spinach (8.2%)	56	0.2449	0.0596	0.00106	—
Turnip greens (4.5%)	84	0.5300	0.3447	0.00410	—
Experiment 8—21 days					
Controls—21 days old	—	0.2172	—	—	—
Calcium carbonate	71	0.4205	0.2033	0.00286	70
Calcium oxalate	56	0.3286	0.1115	0.00199	44
Spinach	37	0.2447	0.0275	0.00074	15
Turnip greens	81	0.4603	0.2431	0.00300	79

RECOVERY OF CALCIUM AND OXALIC ACID IN URINE AND FECES

In a number of these experiments the calcium and oxalate excreted in the urine and feces were determined in addition to the calcium and oxalate in the food consumed and the calcium deposited in the tissue. This included any endogenous oxalic acid which would be overshadowed by the spinach oxalate. The data are recorded in table 4. The collection period was 7 or 8 days duration, thus allowing two periods in a 21-day experiment. The collection was begun 3 or 4 days after the animals were placed on a diet. There seems to be a tendency for less oxalic acid recovery in the second period, indicating that the ability to oxidize it increases with age. Also there is better utilization of calcium in the second period.

While urinary calcium may not seem high with spinach feeding, it represents a relatively high percentage of assimilated calcium, probably passing as oxalate through the excretory organs.

In only one case with spinach in the diet was as much of the calcium utilized as was supplied by the basal diet. The ex-

TABLE 4

Recovery of oxalic acid and calcium

Dietary addition	*Per cent oxalic acid recovered*			*Per cent calcium recovered*			*Calcium utilized*
	In urine	In feces	Total	In urine	In feces	Total	
Experiment 2							
							%
Calcium oxalate	8	39	47				
Spinach	10	23	33				
Experiment 3							
Spinach, Period 1	14	77	91				
Spinach, Period 2	18	72	90				
Spinach + $CaCO_3$, Period 1	12	84	96				
Spinach + $CaCO_3$, Period 2	12	78	90				
Experiment 5							
Spinach, Period 1	14	55	69				
Spinach, Period 2	10	34	44	10	60	70	30
Calcium oxalate, Period 1	6	47	53				
Calcium oxalate, Period 2	6	34	41	3	33	36	64
Experiment 7							
Spinach not drained, Period 1	13	50	63	2	86	88	12
Spinach not drained, Period 2	11	23	34	4	62	66	34
Spinach drained, Period 1	8	57	65	2	57	59	41
Spinach drained, Period 2	6	27	33	3	40	43	57
Experiment 8							
Calcium carbonate, Period 1				18	8	26	74
Calcium carbonate, Period 2				9	6	15	85
Calcium oxalate, Period 1	13	55	68	3	43	46	54
Calcium oxalate, Period 2	6	73	79	3	63	66	34
Spinach, Period 1	11	33	44	3	73	76	24
Spinach, Period 2	8	24	32	8	62	70	30
Turnip greens, Period 1				10	8	18	82
Turnip greens, Period 2				3	6	9	91

ception is in table 4, experiment 7. In this the spinach was cooked by dropping it in twice its weight of boiling water, allowing 7 minutes to return to boiling and then boiling 10 minutes. After draining, its weight was 58% of the original. The undrained spinach was cooked 20 minutes in its adhering water. There was, however, very poor calcium utilization even with drained spinach. Numerous experimental and commercial blanching tests showed it is difficult to remove more than half the oxalate by this process. Spinach calcium is insoluble and hence not extractable.

EXTENDED FEEDING PERIOD

In one experiment twelve animals at 21 days of age were placed on each of two diets. In one group spinach supplied 60% of the calcium and in the other turnip greens. By the time the age of 90 days was reached, five animals on the spinach diet had died while all those on the diet containing turnip greens were in excellent condition. The average weight at 90 days of age of animals receiving spinach was 134 gm. while of those receiving turnip greens it was 205.

In due time two litters of nine young each appeared on the diet with turnip greens. These were reared to the age of 21 days when the average weight was 39 gm. in one litter and 34 in the other, while the average weight of their parents at the same age was 26 gm. Some time later one litter appeared among the animals on the diet with spinach, all but two of which were dead and these were shortly eaten by their mother.

Figures 1, 2 and 3 are photomicrographs of a tooth of an animal respectively on each of the three diets in experiment 3, table 2, i.e., the basal diet alone, with spinach added and with turnip greens. The band across which the line is drawn represents the dentine layer of a rat's tooth magnified 310 times. The dark portion of this band designated 'A' is calcified while the light portion 'B' is uncalcified and represents the area where new dentine is being formed. The wide uncalcified area in figure 2, representing the spinach diet is

apparent. It should be mentioned here that the bones of animals receiving spinach were very soft and pliable.

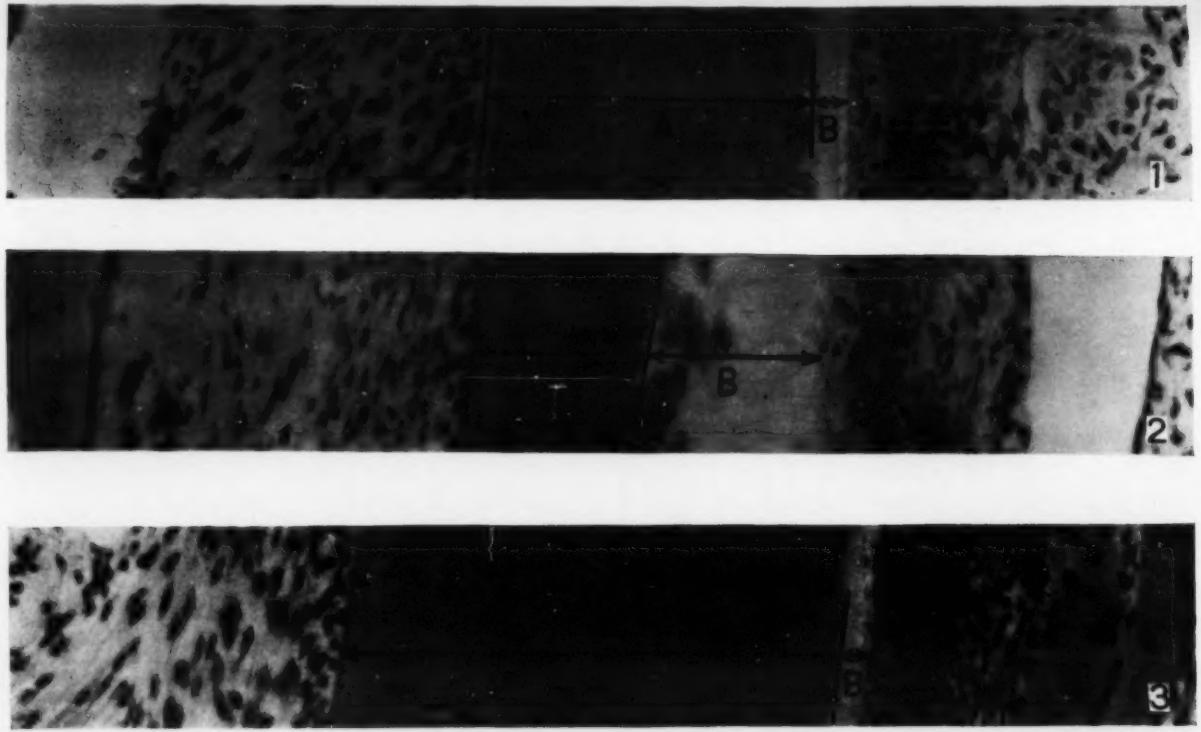
DISCUSSION

Insofar as these data are on a comparable basis they are in agreement with the reports of Tisdall and Drake ('38), Fairbanks and Mitchell ('38) and Fincke and Sherman ('35), published since this work was done. They all dealt with calcium availability. No one seems to have studied heretofore the fate of the oxalates in the diet of the rat. This is perhaps of equal importance. Some oxalic acid is assimilated and tends to carry calcium with it into the urine. In view of the low solubility of calcium oxalate, there arises the question of any possible damage of its passage through the excretory tissues. The demonstration of almost universal presence of small amounts of oxalates in vegetables and fruits tends to throw doubt on the quantitative aspects of endogenous oxalic acid.

CONCLUSION

Oxalates, expressed as anhydrous oxalic acid, have been shown to occur to the extent of about 10% on a dry basis in spinach, New Zealand spinach, Swiss chard, beet tops, lamb's quarter, poke, purslane and rhubarb. Traces were found in nearly all vegetables and fruits.

If to a diet of meat, peas, carrots and sweet potatoes, relatively low in calcium but permitting good though not maximum growth and bone formation, spinach is added to the extent of about 8% to supply 60% of the calcium, a high percentage of deaths occurs among rats fed between the age of 21 and 90 days. Reproduction is impossible. The bones are extremely low in calcium, tooth structure is disorganized and dentine poorly calcified. Spinach not only supplies no available calcium but renders unavailable considerable of that of the other foods. Considerable of the oxalate appears in the urine, much more in the feces.



Photomicrographs, × 310. Dentine of rat's tooth; 'A' calcified, 'B' uncalcified area. Figures 1, 2, and 3 respectively from animal receiving basal diet only, basal diet with spinach and basal diet with turnip greens, diets A, B and C respectively in experiment 3, table 2.

Turnip greens, mustard greens, kale and collards, greens with negligible oxalates, under similar conditions produce excellent animals that deposit four times as much calcium per unit body weight as those receiving spinach.

Acknowledgment is made to Capt. A. L. Irons, Dental Corps, U. S. A., who prepared the photomicrographs, and to the experiment stations at Davis, California, Beltsville, Maryland and Geneva, New York, where various samples were obtained.

LITERATURE CITED

FAIRBANKS, B. W., AND H. H. MITCHELL 1938 The availability of calcium in spinach, in skim milk powder and in calcium oxalate. J. Nutrition, vol. 16, p. 79.

FINCKE, M. L., AND H. C. SHERMAN 1935 The availability of calcium from some typical foods. J. Biol. Chem., vol 110, p. 421.

KOHMAN, E. F., W. H. EDDY AND CELIA ZALL GURIN 1931 A canned food diet. Ind. Eng. Chem., vol. 23, p. 1064.

KOHMAN, E. F., N. H. SANBORN, W. H. EDDY AND CELIA ZALL GURIN 1934 Calcium and vitamin D. Ind. Eng. Chem., vol. 26, p. 758.

KOHMAN, E. F., W. H. EDDY AND MARY E. WHITE 1937 Comparative experiments with canned, home cooked and raw food diets. J. Nutrition, vol. 14, p. 9.

PALKIN, S., A. G. MURRAY AND H. R. WATKINS 1925 Automatic devices for extracting alkaloidal solutions. Ind. Eng. Chem., vol. 17, p. 612.

TISDALL, F. F., AND T. G. H. DRAKE 1938 The utilization of calcium. J. Nutrition, vol. 16, p. 613.

THE DISTRIBUTION OF THE CHICK ANTIDERMATITIS FACTOR (PANTOTHENIC ACID) IN MEATS AND MEAT PRODUCTS[1]

HARRY A. WAISMAN, OLAF MICKELSEN AND C. A. ELVEHJEM
Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison

(Received for publication April 27, 1939)

The chick antidermatitis factor is now recognized as a specific member of the vitamin B complex and distinct from the antipellagra factor. Earlier work by Koehn and Elvehjem ('37) had established that purified liver fractions which prevented the typical dermatitis in chicks were also active in the cure of blacktongue in dogs. Later work by Elvehjem, Madden, Strong and Woolley ('38) showed that the anti-blacktongue activity of these liver fractions was due to the presence of nicotinamide. However, when nicotinic acid or nicotinamide was fed to chicks placed on the heated diet, it was found that these compounds were completely inactive in preventing the typical dermatitis (Mickelsen, Waisman and Elvehjem, '38; Dann and Subbarow, '38). Thus the chick antidermatitis factor is distinct from the antipellagra factor. Recently, in a communication from this laboratory (Woolley, Waisman and Elvehjem, '39), the partial synthesis of the chick antidermatitis factor and its relation to pantothenic acid has been reported. Jukes ('39) has also reported that pantothenic acid is the chick antidermatitis factor. No extensive investigations have been made on the distribution of pantothenic acid in animal tissues, and we wish to report here the distribution of the chick antidermatitis factor in meats and meat products.

[1] Published with approval of the director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the National Live Stock and Meat Board made through the National Research Council.

EXPERIMENTAL

The basal ration 241H used in these assays is composed of yellow corn 58, wheat middlings 25 and crude casein 12; $CaCo_3$ 1; $Ca_3(PO_4)_2$ 1; NaCl 1; hexane extract of alfalfa leaf meal equivalent to 1% of the original meal. The corn, middlings and casein were heated for 30 hours at 120°C. Adequate amounts of vitamin A and vitamin D were provided by biweekly doses with halibut liver oil. The basal ration was supplemented with 100 μg. of thiamin per 100 gm. of ration. Since the quantity of meat which replaced part of the basal furnished some vitamin B_1 itself as shown by work in this laboratory (Mickelsen, Waisman and Elvehjem, '39), the total amount of vitamin B_1 furnished the chicks was more than sufficient for their requirement (Arnold and Elvehjem, '38). In order to insure an adequate riboflavin intake in our rations aside from that furnished by the meat, 200 μg. of crystalline riboflavin (Merck) were added to each 100 gm. of ration. The rations were made up weekly and fed ad libitum.

Day-old White Leghorn chicks were secured from commercial hatcheries and from the Poultry Department of the University. The chicks were placed on raised screens in heated brooders and were weighed weekly. Observations were made daily for lesions on the beak and legs and around the eyes.

The tissues which were used in these assays were of two kinds. One set of samples was obtained from Chicago packers.[2] Samples 1 to 32 were ground vacuum dried, and vacuum packed in tin containers before shipment to us. The remainder of the samples was prepared in our laboratory according to the method of drying described in our earlier publication (Mickelsen, Waisman and Elvehjem, '39). In evaluating the potency of these tissues in the chick antidermatitis factor, we have determined the minimum level of the tissues required to prevent the appearance of lesions in all

[2] We wish to thank Wilson and Company, Armour and Company and Swift and Company for furnishing these samples.

chicks of the group over a 5-week assay period. Although Jukes and Lepkovsky ('36) have described a method for roughly calculating the concentration of the factor in the diet from the growth response, we feel that until a generally accepted standard is available, it is best to report the minimal level of the tissue needed for protection.

In order to determine these minimal levels, the tissues were fed at various percentages of the basal heated diet. It was found that levels of the tissue either higher or lower than the minimal protective level showed corresponding graded increments in growth and incidence of symptoms. Typical results

TABLE 1

Typical increments in growth of chicks fed increasing levels of tissue

TISSUE	SAMPLE NO.	LEVEL FED	AVERAGE WEIGHT AT 5 WEEKS	NUMBER OF BIRDS	NUMBER WITH SYMPTOMS	NUMBER DEAD AT 5 WEEKS
Pork liver	29	0.5	90	5	4	1
	29	1.0	122	4	3	0
	29	2.0	167	4	1	0
	29	4.0	223	8	0	0
Beef lung	78	10	95	4	2	2
	78	20	135	4	1	0
	6	30	172	4	0	0
	6	40	199	4	0	0

obtained with chicks fed these various levels are shown in table 1. When pork liver was fed at 0.5, 1.0, 2.0 and 4.0% levels, much better growth was obtained on the 4% level although complete protection from the typical dermatitis was secured at 2%. The average weight for the four surviving birds on the 0.5% level was 90 gm. at the end of 5 weeks, but all the chicks showed the typical symptoms. The average weight for the chicks on the 1.0% level was 122 gm., but only three out of four birds had the dermatitis. The chicks on the 2% level had an average weight of 167 gm. after 5 weeks and only one bird showed the syndrome. It was found that eight birds on the 4% level averaged 223 gm. and all were completely protected. Another indication of the better growth

obtained on levels of tissue which were higher than that needed for complete protection is that of beef lung. The average weight of the two surviving birds on the 10% level was 95 gm., and these showed the typical dermatitis. The birds on the 20% level averaged 135 gm. after 5 weeks, but only one out of four showed the symptoms. With 30 and 40% levels there was complete protection and growth was increased to 172 gm. and 199 gm. respectively.

Table 2 presents a short summary of the results of the assays for the chick antidermatitis factor (pantothenic acid). Approximately 2% of the liver from the different animals studied was sufficient to protect the chicks. Three samples of beef liver, three of lamb liver, and two samples of veal liver gave protection at the 2% level. The minimal protective level of pork liver must be set at 3% since one out of five birds on the 2% level had the symptoms. It should be pointed out that although 2% of the liver is borderline on the basis of the minimal protective level, the average weight of the birds in the group receiving the 2% pork liver was more than twice that of the basal group at 5 weeks. Pork kidney was active at 1%, while 2% of beef kidney was necessary for complete protection. Lamb kidney was not fed below 5%. Beef spleen was active at 15% while beef brain gave complete protection at 5%. The minimal protective level for beef heart is 5% since all the chicks in the group at this level were entirely free from symptoms, while three out of four chicks on the 3% level exhibited the dermatitis. One of the chicks in the group fed beef lung at a 20% level showed the dermatitis whereas a level of 30% furnished complete protection to the chicks. Two birds out of four in each of two groups receiving 5% and 7.5% respectively of beef pancreas showed the symptoms, thus placing the minimal protective level for pancreas at 10%. Beef tongue gave complete protection at 15%.

In the case of beef muscle there was an indication that the better grade of meat contained more of the chick antidermatitis factor than the meat of lower quality. A sample of sirloin butt (sample 18) from an animal graded at the time

TABLE 2

Summary of the assays for the chick antidermatitis factor

TISSUE	SAMPLE NO.	COMPLETE PROTECTION AT LEVELS	LOWEST LEVEL FED	MINIMAL PROTECTIVE LEVEL
		%	%	%
Beef brain	11	20,30	20	
	57	10,20	10	
	77	5	5	5
Beef heart	13	10,20	10	
	53	10,20	10	
	73	5,10	3	5
Beef kidney	9	3,5	3	
	49	3,5	3	
	84	2,3,5	1	2
Beef liver	10	2,4	2	
	58	2	1	
	98	2,4	1	2
Beef lung	6	30,40	30	
	78		10	25
Beef muscle	18		20	
	19	30	20	
	40		20	
	105	35	25	30
Beef spleen	4	20	10	
	76	20	10	15
Beef pancreas	79	10,20	5	10
Beef tongue	82	15	5	15
Lamb kidney	22	5,10	5	<5
Lamb liver	23	4,10	4	
	61	2,3	2	
	96	1,2	1	1
Lamb muscle	35	30	30	
	80		25	30
Pork kidney	28	3,5	3	
	62	2,4	2	
	83	2	1	1
Pork liver	29	4	0.5	
	86		1	3 or 4
Pork muscle	12	20,30	20	
	24	20,30	20	
	25		20	
	26		30	
	30		20	
	32		20	
	34		20	
	51	30	20	
	47	20,30	20	20–30
Veal muscle	16	40	40	
	44	25,35	25	
	75		25	25–35
Veal liver	5	4	2	
	97	2	1	2
Stewed beef heart	87	7.5,10	5	7.5
Stewed beef spleen	88		5	15
Stewed beef kidney	85	5	3	5
Fried beef liver	99	1,2,4		1

of slaughter as fair did not offer complete protection at a level of 20% whereas a similar cut from a prime steer (sample 19) did give protection at the same level. A third sample of prime beef (sample 40) also offered protection at a 20% level. Still another sample of beef (sample 105) gave protection at a level of 25%. One sample of lamb muscle (sample 80) did not offer protection at either a level of 25% or 30%, but 30% of sample 35 did give complete protection and good growth.

Nine different samples of pork muscle were assayed and in general it appears that the minimal protective level is between 20 and 30%. There was considerable variation in the potency of these samples. Pork loin (sample 30) did not protect the chicks from dermatitis nor did it produce good growth when fed at levels as high as 30%, whereas sample 24 at a level of 20% afforded protection and induced fair growth. A sample of pork shoulder (sample 25) and one of pork ham (sample 26) produced borderline protection at levels of 20 and 30% respectively as shown by the fact that one chick in each of these groups had slight symptoms of dermatitis. Another sample of pork ham (sample 51) did not give complete protection at the 20% level but did give it at the 30% level. Three samples of veal muscle also showed variations in their ability to prevent chick dermatitis. Sample 44 afforded complete protection at a level of 35% while sample 75 at the same level was not able to protect the birds completely. Sample 16 at a level of 40% gave complete protection and good growth.

Although it had previously been shown that the chick antidermatitis factor was labile to dry heating but stable to autoclaving, no reports have appeared on the influence of cooking on this factor. A few cooked samples described in our earlier paper (Mickelsen, Waisman and Elvehjem, '39) and a sample of fried beef liver were assayed in an effort to determine the destruction produced by these methods of cooking. The stewed beef heart had to be fed at a level of 7.5% in order to produce complete protection while the uncooked beef heart was active at a level of 5%. Stewed beef kidney was protective at a level of 5% which is in contrast to the activity of the

uncooked sample at a level of 2%. The sample of stewed beef spleen was fed at a level of 15% in order to produce complete protection whereas the uncooked sample was active at a level of 10%. We have found that fried beef liver fed at a 1% level protects the chicks from dermatitis but does not allow good growth. When this sample was fed at a 2% level there was complete protection and good growth, indicating that under the method of frying employed, there was no destruction of the factor. It is of interest to note that we have observed slightly better growth on the 2% of fried beef liver than on 2% of the untreated tissue of the same animal. In one series the average weight of the birds receiving the untreated liver was 172 gm. at 5 weeks, while that of the birds receiving the fried liver was 263 gm. A comparison of the average weight of twelve chicks on 2% of untreated liver with the average weight of ten chicks on the fried liver gives 202 grams and 241 gm. respectively.

DISCUSSION

The results of these assays indicate that liver and kidney from various animals are two of the richest animal sources of the chick antidermatitis factor. A level of 2% will furnish complete protection and produce good growth. Beef heart and beef brain are next in potency with the minimal level at 5% while between 10 and 15% of beef spleen are required for protection. Beef pancreas is protective at 10% and beef tongue is active at the 15% level. In contrast to these values the muscles of pork, beef, lamb and veal are required at levels between 25 and 35% before any protection is obtained. Beef lung was also required at a level between 25 and 30% before it gave complete protection. Jukes and Lepkovsky ('36) found that 24% of meat scraps was a poor source of the filtrate factor. Jukes ('37) fed vacuum dried beef round at 25 and 50% levels and concluded that it had a low filtrate factor value. We have found that the minimal protective level for beef muscle is 25% and is of much lower potency when compared to the organs of the same specie.

The influence of stewing on the chick antidermatitis content of beef heart, beef spleen and beef kidney is readily brought out by a comparison of the untreated and cooked samples. It can be concluded that approximately one-third of the potency was lost by this method of cooking. This decreased potency of the cooked meats can be best explained by the partial extraction of the factor by the water in which the meat was cooked. The influence of frying on the potency of liver is evidently not of significance since the chicks were protected from symptoms at the 1 and 2% levels. At present it is difficult to explain the increased growth obtained in the groups receiving the fried liver over that of those receiving the uncooked tissue. This finding was consistent in a number of trials and cannot be attributed to a fortuitous choice of chicks.

It is interesting to consider the variations in the amount of the various water soluble vitamins in the same tissue. A comparison of the distribution of the chick antidermatitis factor with that of vitamin B_1 indicates that the highest concentration of vitamin B_1 was found in pork ham, yet this tissue was lowest in the chick antidermatis factor. The values for liver and kidney also demonstrate the wide differences in the occurrence of the two factors. Here again the liver and kidney are comparatively low in vitamin B_1 when compared to other tissues and are the richest in the chick antidermatitis factor. We found very little variation in the chick antidermatitis factor content of these tissues obtained from different species. The minimal protective level for pork, beef and veal liver was 2% while the muscle tissue from lamb, beef, pork and veal offered protection at levels between 25 and 30%.

Work from this laboratory (Peterson and Elvehjem, '39) indicates that from 2 to 6% of dried yeasts grown on various media will give complete protection from dermatitis in chicks fed the heated basal diet. Dried liver and kidney can be considered equally good sources of the chick antidermatitis factor (pantothenic acid) since these tissues are also active at the 2% level.

The use of our heated basal diet 241H was fully satisfactory for the assay of the chick antidermatitis factor (pantothenic acid). We have obtained growth with this diet when a good source of the chick factor was supplied. However, an interesting observation was made on the growth of the chicks receiving certain levels of the tissues. Fairly good growth was observed in some of the groups even though the chicks showed typical dermatitis. When 1% of beef liver was fed to one group, the four birds showed the syndrome but the average weight reached 115 gm. at 5 weeks which is nearly twice that of the chicks on the basal diet. In the case of 10% beef spleen (sample 76) the average growth was 154 gm. but three birds showed dermatitis. Typical lesions were also observed in three of the chicks getting 5% beef tongue (sample 82) while the average growth was 117 gm. These few examples are sufficient to suggest a possible distinction between the chick antidermatitis factor and a growth-promoting substance. We cannot say definitely at present that the factor which protects against dermatitis in chicks will also stimulate growth or that the two factors are distinct. While our work was in progress, Bauernfeind et al. ('38) indicated that a new factor was required for growth and reproduction in the domestic fowl. They found that the factor is destroyed by the method of preparation of the heated diet. Although the minimal protective levels of our tissues protected the chicks from symptoms and at the same time produced good growth, the lower levels of the same tissues frequently produced much better growth than that obtained on the basal ration even though the birds showed dermatitis. This is evidence that the heated diet is probably deficient in a factor other than that which protects against chick dermatitis.

SUMMARY

1. The minimal protective levels for the chick antidermatitis factor (pantothenic acid) in meat and meat products have been determined.

2. The liver and kidney of various species were found to be the richest sources of the factor, followed by heart, spleen, brain, pancreas, tongue and lung. The muscular tissues of beef, lamb, pork and veal had the least potency.

3. The ordinary household method of stewing decreased the potency of kidney, heart and spleen by one-third.

4. The factor in liver was not destroyed by frying. On the contrary, better growth was consistently obtained with the fried beef liver than with the uncooked tissue.

LITERATURE CITED

ARNOLD, A., AND C. A. ELVEHJEM 1938 Studies on the vitamin B_1 requirements of growing chicks. J. Nutrition, vol. 15, p. 403.

BAUERNFEIND, J. C., A. E. SCHUMACHER, A. Z. HODSON, L. C. NORRIS AND G. F. HEUSER 1938 New factor required for growth and reproduction in the domestic fowl. Proc. Soc. Exp. Biol. and Med., vol. 39, p. 108.

DANN, W. J., AND Y. SUBBAROW 1938 Differentiation of the rat dermatitis factor and the chick dermatitis factor from nicotinic acid. J. Nutrition, vol. 16, p. 183.

ELVEHJEM, C. A., R. J. MADDEN, F. M. STRONG AND D. W. WOOLLEY 1938 The isolation and identification of the anti-blacktongue factor J. Biol. Chem., vol. 123, p. 137.

JUKES, T. H. 1937 Further observations on the assay, distribution and properties of the filtrate factor. J. Biol. Chem., vol. 117, p. 11.

———— 1939 Pantothenic acid and the filtrate (chick antidermatitis) factor. J. Am. Chem. Soc., vol. 61, p. 975.

JUKES, T. H., AND S. LEPKOVSKY 1936 Distribution of the 'filtrate factor' (a water-soluble vitamin belonging to the vitamin B complex and preventing a dietary dermatitis in chicks) in certain feedingstuffs. J. Biol. Chem., vol. 114, p. 117.

KOEHN, C. J., JR., AND C. A. ELVEHJEM 1937 Further studies on the concentration of the antipellagra vitamin. J. Biol. Chem., vol. 118, p. 693.

MICKELSEN, O., H. A. WAISMAN AND C. A. ELVEHJEM 1938 Inactivity of nicotinic acid in chick dermatitis. J. Biol. Chem., vol. 124, p. 313.

———— 1939 Distribution of vitamin B_1 (thiamin) in meat and meat products. J. Nutrition, vol. 17, p. 269.

PETERSON, W. H., AND C. A. ELVEHJEM 1939 Conditions affecting the content of chick antidermatitis vitamin in yeast. (In press.)

WOOLLEY, D. W., H. A. WAISMAN AND C. A. ELVEHJEM 1939 Nature and partial synthesis of the chick antidermatitis factor J. Am. Chem. Soc., vol. 61, p. 977.

ON THE APPROXIMATION OF THE CALCULATED TO DETERMINED CALCIUM CONTENT OF HUMAN DIETARIES

ALEXANDER B. GUTMAN AND MARGARET LOW

Department of Medicine, College of Physicians and Surgeons, Columbia University and the Department of Nutrition, Presbyterian Hospital, New York City

(Received for publication May 12, 1939)

It is a matter of some practical importance to determine how closely the composition of diets can be approximated by calculations based upon tables giving mean analytical values for each component foodstuff. This applies particularly to the calcium content of human dietaries since it is known that the coefficient of variation with respect to the calcium content of many food stuffs is conspicuously large (Sherman, '37). Where analyses of diets for calcium content have been made and the results compared with values computed by reference to various tables (Bauer and Aub, '27; Bassett and van Alstine, '35), the determined values have been found to be significantly and quite consistently lower than the calculated estimates. A discrepancy in the same direction and of similar degree was noted by us in the course of our own metabolic studies.

Sherman's continued investigations of the composition of foods have led to revision of the mean calcium content of certain foodstuffs (Sherman, '37). It seemed to us of interest to compare the determined with the calculated calcium content of a number of representative diets, utilizing the revised data recently made available. As the validity of such comparisons depends, in part, upon the variation inherent in our methods of analysis, we have summarized in table 1 our results of determinations made in consecutive periods of

representative diets. Table 1 indicates the range of variation in found values attributable to variations in the calcium content of raw foodstuffs plus technical errors in sampling, due to losses in cooking of foods and in chemical analysis. It should be emphasized that the determined values used for comparisons with Sherman's figures for raw foodstuffs (Sherman, '26, '37) are the results of analysis of cooked foods

TABLE 1

Results of calcium analyses of diets in consecutive 5-day periods showing scatter due to Σ variations in 1. Ca content of raw foodstuffs, 2. sampling, 3. losses in cooking, 4. chemical analyses

Diet	Period	Determined Ca content of diet (mg. per day)	Deviation from mean (mg.)	Diet	Period	Determined Ca content of diet (mg. per day)	Deviation from mean (mg.)
A	1	911	−39	E	1	473	+ 5
	2	945	− 5		2	460	− 8
	3	994	+44		3	468	± 0
	Mean = 950				4	448	−20
B	1	926	+55		5	494	+26
	2	848	−23		6	460	− 8
	3	892	+21		7	472	+ 4
	4	878	+ 7		Mean = 468		
	5	810	−61	F	1	466	+14
	Mean = 871				2	450	− 2
C	1	1072	−27		3	446	− 6
	2	1106	− 3		4	432	−20
	3	1240	+131		5	448	− 4
	4	1068	−41		6	468	+16
	5	1088	−21		7	456	+ 4
	6	1122	+13		8	452	± 0
	7	1066	−43		Mean = 452		
	Mean = 1109			G	1	678	− 2
D	1	465	+21		2	696	+16
	2	452	+ 8		3	674	− 6
	3	474	+30		4	690	+10
	4	438	− 6		5	746	+66
	5	410	−34		6	668	−12
	6	448	+ 4		7	660	−20
	7	422	−22		8	670	−10
	Mean = 444				9	668	−12
					10	656	−24
					11	678	− 2
					Mean = 680		

prepared under conditions designed to minimize leaching out of minerals.

METHODS

The general procedure outlined by Bauer and Aub ('27) was followed in our mineral balance studies, which were conducted in the metabolism wards of the Presbyterian Hospital under the supervision of a specially-trained personnel. The dietaries were calculated and the raw foods weighed and prepared in a diet kitchen adjoining the metabolism ward, under the direction of the dietitian in charge. In order to minimize variations in composition, quantities of raw food sufficient for one 5-day period (or two 3-day periods, in some instances) plus enough food for 1 extra day were purchased from uniform sources at the beginning of the metabolic period. Foods subject to quick deterioration were divided into two portions, the part not required for immediate use being stored in a special low-temperature refrigerator. In order to minimize loss of mineral content, the food was weighed, cooked and served in the same dish; in the case of vegetables and fruits (when not served raw) the cooking being done in high glazed, covered casseroles by pressure without addition of water. Meats were broiled on a large-mesh wire grill over a casserole to catch drippings and were served in that casserole. The extra day's aliquot of food was weighed and prepared exactly as for the trays tendered daily to the patient but was submitted to the laboratory for analysis. There the foods with their juices were removed quantitatively, minced and thoroughly mixed by hand at first, more recently with a meat grinder and mechanical stirrer. The food mass was dried, pulverized and triplicate 1 to 2 gm. samples removed for ashing in a muffle furnace. After treatment of the ash in the manner described by Tisdall and Kramer ('21), calcium was determined by the Clark and Collip ('25) modification of that method.

RESULTS

Table 2 summarizes comparisons between determined values for the calcium content of diets and values calculated by reference to tables published by Sherman in 1926 and to the revised 1937 figures. The data comprise twenty-eight observations made in the course of metabolic studies on twenty-one cases during the period 1930 to 1939. As the comparisons appear to vary significantly according to the dietary calcium level, they are divided into two groups: high and intermediate calcium diets containing more than 400 mg. per day, and low calcium diets containing less than 200 mg. per day. The data in each group are arranged in order of increasing negativity of the value determined minus calculated (by 1937 figures), expressed in percent of the calculated estimate. The month and year of each observation are recorded though no definite seasonal trend could be recognized.

The point of major interest in these results (table 2) concerns the consistency and degree of the positive deviation from determined values observed in calculated estimates, whether based upon the 1926 or upon the revised 1937 figures. The calculated estimates exceeded determined values more consistently in low calcium diets (in fourteen of sixteen observations) than in intermediate or high calcium diets (eight of twelve observations using the 1937 figures). In these latter diets, too, the scatter in percentage deviations of the calculated values was found to be smaller, showing a total range of +13 to −24% as compared with +16 to −49%. The absolute error in milligrams calcium, however, may be much larger in high than in low calcium diets.

The use of Sherman's revised figures for the mean calcium content of foodstuffs invariably yielded lower net values for total calcium content than were obtained with the use of earlier figures. The difference was small in low calcium diets where it was due almost entirely to lower mean values for orange and other fruit juices.

In general, the revised figures appear to afford better agreement between determined and calculated values for the cal-

TABLE 2

Comparison of determined with calculated values for the calcium content of human dietaries

Case	Date	Calcium in diet (mg. per day) Determined	Calculated (Sherman 1936)	Calculated (Sherman 1937)	Determined minus calculated (Sherman 1936)	Determined minus calculated (Sherman 1937)	Det. minus calculated (S 1937)
					mg.	mg.	%
I. High and intermediate calcium diets							
Mc.	III-31	923	840	803	+ 83	+120	+13
Oc.	IV-31	1109 [1]	1041	1001	+ 68	+108	+10
Wo.	XII-32	992	1006	941	— 14	+ 51	+ 5
Wo.	XII-32	981	1006	941	— 25	+ 40	+ 4
St.	I-37	680 [1]	715	688 [2]	— 35	— 8	— 1
Mm.	V-37	1716	2589 [3]	1765		— 49	— 3
Pa.	XI-36	871 [1]	952	906	— 81	— 35	— 4
Mk.	X-37	2010	2259	2105	—249	— 95	— 5
Fl.	X-36	452 [1]	541	507 [2]	— 89	— 55	—12
Bo.	II-38	444 [1]	566	530	—122	— 86	—19
Mm.	V-37	1178	1560	1473	—382	—295	—20
Bo.	I-38	468 [1]	644	579	—176	—111	—24
II. Low calcium diets							
No.	II-36	126	113	109	+ 13	+ 17	+16
Mo.	X-38	138	136	131	+ 2	+ 7	+ 5
Sc.	VII-38	157	180	168	— 23	— 11	— 7
Gr.	VIII-33	118	131	128	— 13	— 10	— 8
Li.	VIII-36	96	119	115	— 23	— 19	—17
Li.	VIII-36	94	119	115	— 26	— 21	—18
Mh.	XI-37	128	174	173	— 46	— 45	—26
De.	V-37	115	169	161	— 54	— 46	—29
Ba.	VI-37	126	210	183	— 84	— 57	—31
We.	IV-38	110	180	163	— 70	— 53	—32
Sa.	IV-33	80	131	127	— 51	— 47	—37
Mm.	IV-37	70	119	115	— 49	— 45	—39
Mm.	IV-37	69	119	115	— 50	— 46	—40
Wo.	XII-32	73	130	126	— 57	— 53	—41
Gu.	IV-38	100	183	171	— 83	— 71	—42
Le.	II-39	44	101	86	— 56	— 42	—49

[1] Mean of several periods (see table 1).

[2] Milk powder (Dryco) used instead of milk and calculated 10 gm. Dryco = 95 gm. skimmed milk.

[3] Cottage cheese calculated as hard cheese.

cium content of diets than do the older figures. This obtained, for example, in twenty-two of the twenty-eight diets in our series. However, even with the use of the revised figures the calculated estimates were still too high in twenty-two of the twenty-eight diets studied; in our sixteen low calcium diets the positive deviation exceeded 25% of the calculated value in ten instances. Loss of calcium in cooking may account for part of this discrepancy. But because of the precautions taken in this regard, we doubt that the discrepancy can be explained wholly in this way, particularly in the low calcium diets in which the major portion of the calcium content was supplied by uncooked foods.

Our figures emphasize again the hazards of employing calculated estimates of the calcium content of diets when conducting calcium balances for clinical or metabolic studies. Variables affecting the absorption and utilization of calcium from dietary sources constitute further sources of error not indicated by our analyses.

SUMMARY

The calcium content of sixteen low calcium diets and twelve intermediate and high calcium diets was determined by analysis. The results were compared with the dietary calcium content as computed by reference to tables giving mean calcium contents of the component foodstuffs. In twenty-two of twenty-eight observations, Sherman's revised ('37) tables gave better agreement between calculated and found values than did earlier figures. A disproportionate number of calculated estimates, however, whether based upon earlier or upon revised data, exceeded the determined values, the positive deviation being greater than 25% of the calculated value in ten of sixteen low calcium diets.

LITERATURE CITED

BASSETT, SAMUEL H., AND HELEN E. VAN ALSTINE 1935 Mineral exchanges of man. IV. Variations in the mineral content of diets with a constant raw weight formula. J. Nutrition, vol. 9, p. 175.

BAUER, WALTER, AND JOSEPH C. AUB 1927 Studies of inorganic salt metabolism. I. The ward routine and methods. J. Am. Diet. Assoc., vol. 3, p. 106.

CLARK, E. P., AND J. C. COLLIP 1925 A study of the Tisdall method for the determination of blood serum calcium with a suggested modification. J. Biol. Chem., vol. 63, p. 461.

SHERMAN, H. C. 1926 Chemistry of food and nutrition, 3rd ed. The Macmillan Co., New York.

———— 1937 Chemistry of food and nutrition, 5th ed. The Macmillan Co., New York.

TISDALL, FREDERICK F., AND BENJAMIN KRAMER 1921 Methods for the direct quantitative determination of sodium, potassium, calcium and magnesium in urine and stools. J. Biol. Chem., vol. 48, p. 1.

THE RAT GROWTH FACTORS OF THE FILTRATE FRACTION OF LIVER EXTRACTS[1]

GEORGE H. HITCHINGS AND Y. SUBBAROW

Department of Physiology, Harvard School of Public Health and Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts

ONE FIGURE

(Received for publication May 4, 1939)

The B group of vitamins has been shown to contain one or more unknown substances in addition to the factors thiamin, riboflavin and vitamin B_6, which already have been isolated (Schultz, '37, '38; Lepkovsky, '36; Frost, '37; Edgar, '37). Both yeast (Edgar, '37; Schultz, '37, '38) and liver extracts (Lepkovsky, '37; Oleson, '39; Frost, '39; Edgar, '38 a) have been used as sources of the unknown substances. The extracts from the two sources have been found to have similar, but not identical, properties. Specifically, Edgar and Macrae ('37) reported that treatment of yeast extracts with fuller's earth results in a sharp partition of the rat growth-promoting activity. When given singly, neither the filtrate nor the adsorbate has more than slight activity; but when both are given, complete growth results. Edgar, El Sadr and Macrae ('38 a) found that fuller's earth treatment did not yield inactive fractions when applied to liver extracts. However, fractions with the same biological properties as the yeast eluate and filtrate fractions could be prepared by other means.

On the other hand, Oleson, Bird, Elvehjem and Hart ('39) report that when their liver extracts are treated with fuller's earth, neither the filtrate nor the eluate actively promotes

[1] These investigations have been supported in part by the Ella Sachs Plaut Foundation and the Proctor Fund of Harvard University.

growth. Moreover, when both fractions are given, the animals fail to grow normally, although symptoms of deficiency diseases are prevented.

The present paper is concerned with the study of the rat growth-promoting substances of the so-called '95% alcoholic filtrate' of liver (Cohn fraction F, '27). This starting material, on treatment with fuller's earth, yields two fractions, both of which are relatively inactive when given separately but in combination have the full activity of the original extract. The 95% alcoholic filtrate, therefore, is a convenient starting material for a study of the growth-promoting substances of the fuller's earth filtrate. A report of the progress made on this problem follows.

EXPERIMENTAL

Diet. The basal diet (BF) used consists of the following: casein 200, peanut oil 80, cod liver oil 20, sucrose 120, cornstarch 500, wood flour 40, salts (Osborne and Mendel, '19) 40. The casein is heated on the water bath under reflux condenser with 2.5 parts of 95% ethanol for a period of 6 hours; this procedure is repeated twice.

For most of the experiments reported herein, the basal diet was supplemented by the addition of fuller's earth adsorbate (S4). When used, 5.4 gm. of fuller's earth adsorbate, prepared as described below, was incorporated in 1 kg. of the basal diet.

Thiamin and riboflavin were given in doses of 70 and 80 γ, respectively, three times each week to all animals.

The various liver fractions under investigation were administered orally from a 1 cc. calibrated hypodermic syringe in equal doses (usually 0.2 cc.) three times each week.

Experimental animals. All the albino rats used for the experiment were from inbred stock of The Wistar Institute strain. The mothers and young were placed on the basal diet of the experiment (either S4 or BF) when the young reached a weight of 30 to 35 gm., and the young were weaned and isolated when they reached a weight of 40 to 50 gm. at about

24 days of age. Unless otherwise noted, each test group contained six animals, three males and three females, in most instances each pair coming from a different family.

Preparation of liver extract fractions

Commercial 95% alcoholic filtrate, a by-product in the preparation of the pernicious-anemia curative material in pig's liver extract, was used as the starting material for all supplements. Each cubic centimeter of this solution represents material from aproximately 100 gm. of raw liver. The concentration of each of the various fractions has been referred to the quantity of original liver represented in the starting material.

Fuller's earth filtrate and adsorbate. The 95% alcoholic filtrate was treated at pH = 1 with 0.5 gm. of fuller's earth for each 100 gm. of liver represented in the solution. After stirring for about 30 minutes, the solution was filtered and the adsorbate was washed with water and air-dried. This adsorbate was used without elution as the S4 supplement. An aliquot of the filtrate was diluted to a concentration equivalent to 60 gm. of liver per cubic centimeter. This has been designated as fraction S2.

The procedures used for concentrating the active substance of the fuller's earth filtrate are illustrated in chart 1.

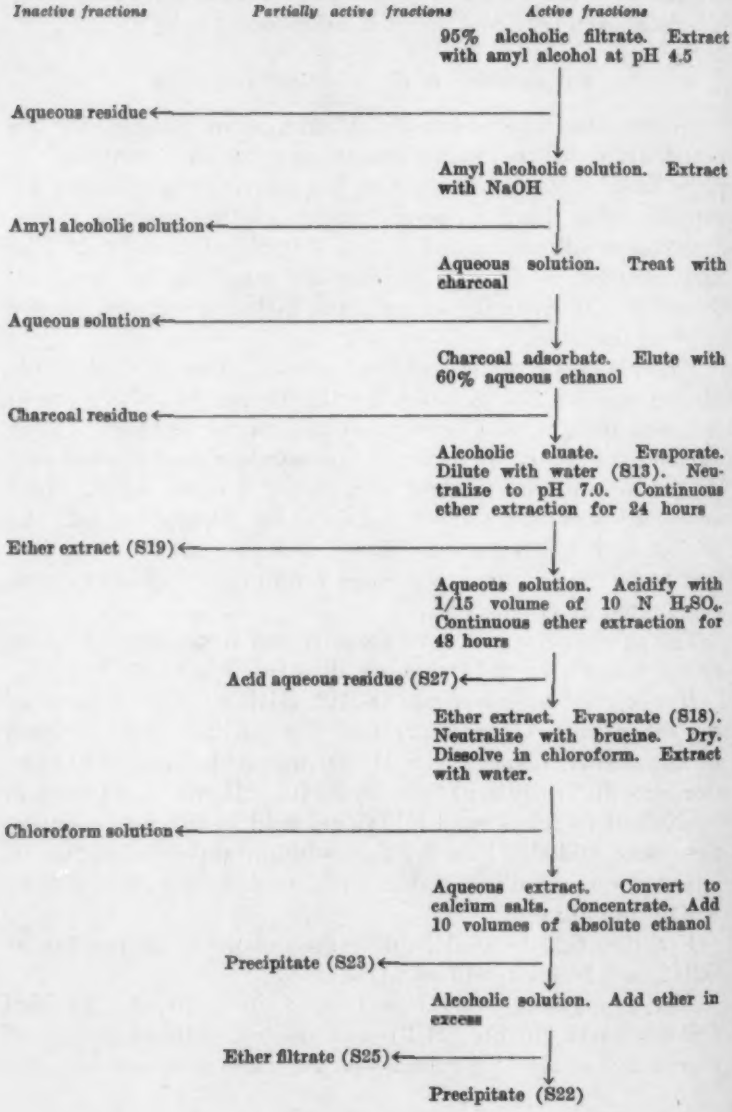
Hydrolyzed preparations (S2H2, S1H3). To solutions of the ether precipitate (S22) and the purified fuller's earth filtrate (S13), respectively, H_2SO_4 was added until the solution was distinctly acid to thymol blue. H_2SO_4 in an amount equivalent to 0.2 normal additional acid in the final solution then was added. Finally, the solution was heated for 60 minutes on a boiling water bath, cooled, neutralized with NaOH and filtered.

Fraction S22 treated as described above is designated as S2H2, and fraction S13 as S1H3.

Mercury filtrate (S33) *and precipitate* (S30). Purified fuller's earth filtrate (S13) was treated with an excess of mercuric acetate (1.2 volumes of 20% mercuric acetate solu-

CHART 1

Steps in the concentration of the growth-promoting substances of liver extracts


Inactive fractions
Partially active fractions
Active fractions
95% alcoholic filtrate. Extract with amyl alcohol at pH 4.5
Aqueous residue
Amyl alcoholic solution. Extract with NaOH
Amyl alcoholic solution
Aqueous solution. Treat with charcoal
Aqueous solution
Charcoal adsorbate. Elute with 60% aqueous ethanol
Charcoal residue
Alcoholic eluate. Evaporate. Dilute with water (S13). Neutralize to pH 7.0. Continuous ether extraction for 24 hours
Ether extract (S19)
Aqueous solution. Acidify with 1/15 volume of 10 N H_2SO_4. Continuous ether extraction for 48 hours
Acid aqueous residue (S27)
Ether extract. Evaporate (S18). Neutralize with brucine. Dry. Dissolve in chloroform. Extract with water.
Chloroform solution
Aqueous extract. Convert to calcium salts. Concentrate. Add 10 volumes of absolute ethanol
Precipitate (S23)
Alcoholic solution. Add ether in excess
Ether filtrate (S25)
Precipitate (S22)

tion) and the mixture was allowed to stand overnight in the refrigerator. The mixture was centrifuged, and the precipitate was washed once with water. The precipitate and the combined filtrate and washings were decomposed in the cold with H_2S. The solutions were aerated, filtered and the filtrates were neutralized with sodium hydroxide solution.

RESULTS

The results obtained in testing the biological activity of the various fractions described in the preceding section are presented in table 1. The results have been expressed as the average gain in weight per week for each group (six rats except where otherwise stated). Most tests were run for at least 5 weeks. The average gain per week for a 5-week period has been tabulated in the last column and forms the basis for evaluating the relative activity of each fraction. In general, fractions were considered active when the rate of gain in weight exceeded that of the controls by 50%.

In order to illustrate the differences in rates of growth obtained with different fractions, examples of the experiments with fractions S4, S13 and S22 have been plotted in figure 1. The significance of the results will be discussed subsequently.

It should be noted that even with the addition of supplements which give excellent initial weight increases, the rate of gain is not maintained throughout the 5-week period. This suggests that even the crude fractions S2 and S4 do not contain all the substances essential for sustained growth in optimum amounts (v.i., p. 273).

The basal diet without added liver supplement supported a negligible increase in weight. Addition of the fuller's earth filtrate (S2) alone gave an average weight increase of 9.3 gm. per week and of the fuller's earth adsorbate (S4) alone gave an increase of 6.7 gm. per week. Administering the two together gave a weight increase of 18.4 gm. per week. Therefore, progress in concentrating the active material of the fuller's earth filtrate could be followed by measuring the

growth obtained upon administering the various fractions to animals receiving S4 in the diet.

Using these criteria it was found that the growth-promoting factors could be shaken out into amyl alcohol from acid solution, taken back into dilute sodium hydroxide, adsorbed on carbon and eluted with aqueous ethanol without great loss (S13). From this preparation part of the activity could be

TABLE 1

Biological response to various liver extract fractions added to basal diet BF

SUPLEMENT ADDED TO BASAL DIET			AVERAGE GAIN IN WEIGHT EACH WEEK					AVERAGE GAIN PER WEEK 5-WEEK PERIOD
Experiment no.	Description	Amount as liver equivalent in grams per week per rat [2]	1	2	3	4	5	
			gm.	gm.	gm.	gm.	gm.	gm.
..	None	..	0.0	0.3	0.0	0.3	−0.5	0.0
S2	Fullers' earth filtrate	36	8.0	9.2	9.3	9.3	10.9	9.3
S4	Fullers' earth adsorbate	60–90	7.2	9.0	7.5	6.0	3.8	6.7
S2+S4	Fullers' earth filtrate	36	18.3	17.9	20.7	20.2	14.9	18.4
S13+S4	Purified F.E.F.	60	25.0	20.7	20.0	17.0	14.6	19.5
S19+S4	Neutral ether extract	140	4.8	6.0	7.0	8.0	8.0	6.8
S18+S4	Acid ether extract	140	20.0	16.5	15.0	..	..	(17.2)
S18+S4	Acid ether extract	690	22.5	25.5	17.0	12.2	..	(19.3)
S27+S4	Residue from ether extraction	140	8.2	10.8	15.0	11.5	11.3	11.4
S23+S4[1]	Alcohol precipitate	640	9.8	16.8	19.5	11.5	..	(14.4)
S22+S4[1]	Ether precipitate	320	20.5	15.8	17.3	11.3	9.8	14.9
S22+S27+S4[1]		[3]	16.3	18.7	22.2	11.7	12.8	16.3
S25+S4	Ether filtrate	580	9.5	16.5	14.5	12.3	..	(13.2)
S2H2+S4	S22 hydrolyzed	290	7.8	4.3	6.0	8.5	8.5	7.0
S1H3+S4	S13 hydrolyzed	60	10.5	14.7	13.7	12.9	11.9	12.7
S33+S4	Mercury filtrate	60	12.8	23.2	20.2	15.8	6.8	15.8
S30+S4	Mercury precipitate	60	11.3	10.0	6.8	7.7	..	(9.0)
S33+S30+S4		[3]	15.5	20.8	19.0	17.8	9.2	16.5
S19+S22+S4		[3]	14.7	19.0	20.2	11.8	11.0	15.3

[1] Four rats instead of six were in each of these series.

[2] This refers to the amount of supplement other than S4, which when supplied was constant throughout.

[3] Each supplement was administered in an amount equal to that shown for the same supplement when given above.

extracted by ether from acid (S18) but not from neutral (S19) solution. The procedure was attended by considerable loss indicated by the increase in the amount of liver equivalent needed to promote rat growth. Neither the extract (S18) nor the residue (S27) is as active as the original material (S13).

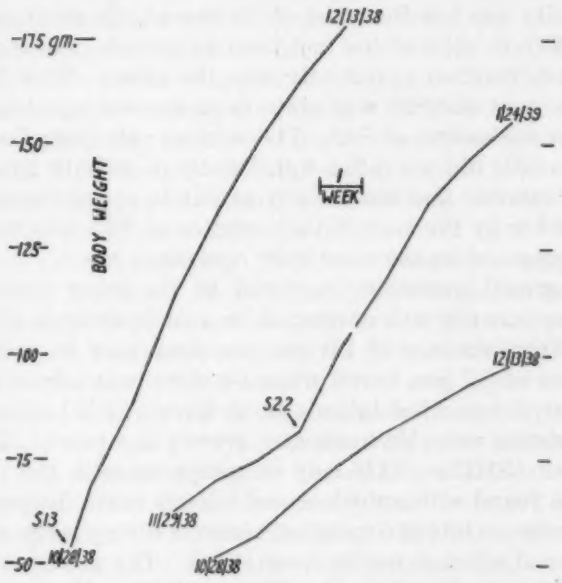


Fig. 1 Examples of average weight curves of rats given various liver extracts as supplements to the basal diet. Experiments with S13 + S4 and S4 were each carried out with a group of six rats; the experiment with S22 + S4, with a group of four rats. All animals had received the supplement S4 since weaning. The other supplements were added at the times indicated by the arrows.

Moreover, when the most active fraction of the ether-extractable material is given along with the residue (S22 + S27) the activity of the original extract is not reached even when both fractions are given in greater amounts. The ether extraction, however, serves the useful purpose of separating much of the active substance from a large amount of inert material.

When the brucine salts, from S18, are partitioned between water and chloroform, the greater part of the activity goes into the aqueous phase. This was shown by determining the activity contained in the calcium salts of the fractions S23, S22 and S25. The first of these, the alcoholic precipitate (S23), showed some growth-promoting activity. However, its activity was less than that of the second, the ether precipitate (S22), in spite of the fact that the growth rate per week with each fraction apparently was the same. This follows from the fact that S23 was given in an amount equal to twice the liver equivalent of S22. The calcium salt from the ether filtrate (S25) did not differ significantly in activity from S23. It is of interest that the activity of S18 is approximately accounted for by the sum of the activties of S22, S23 and S25 when compared on the same liver equivalent basis.

The growth-promoting material of the ether precipitate (S22) apparently was destroyed by acid hydrolysis (S2H2). The weight increase of 7.0 gm. per week may be compared with that of 6.7 gm. found when S4 alone was administered. However, the purified fuller's earth filtrate (S13) after similar treatment was able to support growth at a rate of 12.7 gm. per week (S1H3). This may be compared with the rate of 19.5 gm. found with unhydrolyzed fuller's earth filtrate.

Mercuric acetate precipitation removes a very large amount of material which is nearly inert (S30). The greater part of the activity is found in the filtrate (S33). Moreover, the growth rate when both fractions are given is not significantly greater than that of the mercury filtrate alone.

The neutral ether extract (S19) was not only inactive of itself, but also failed to supplement in any way the activity of the ether precipitate (S19 + S22).

DISCUSSION

It is obvious that we are dealing with the same group of factors as Edgar, Macrae and co-workers. The successful adsorption of the activity on charcoal corresponds with the experience of Edgar and Macrae ('37) with the yeast filtrate

factor. The extraction of the activity from acid solution by amyl alcohol, and its return into dilute aqueous sodium hydroxide is analogous to the preparation of the liver filtrate factor by Edgar, El Sadr and Macrae ('38 b). The failure of mercuric acetate to precipitate any significant part of the liver filtrate activity (S33 + S30) is similar to the behavior of yeast filtrate factor ('37).

On the other hand, differences between the findings of the two laboratories have appeared. The growth rates reported by Edgar, Macrae and co-workers, with both yeast and liver supplements, are consistently higher than those which we find with the 95% alcoholic filtrate [2] and are about the same magnitude as those we find with cruder extracts. Moreover, there is a difference in the behavior of the liver extracts toward fuller's earth treatment. Together these facts suggest that the Liver Residue I of Edgar, El Sadr and Macrae ('38 b) may contain another accessory growth substance present in relatively low concentration in the 95% alcoholic filtrate.

The extraction of the activity from acid aqueous solution represents a new step in the concentration of the material necessary for rat growth. Continuous ether extraction recently was successfully applied by Elvehjem and collaborators to the problem of the chicken antidermatitis factor (Woolley, '38). However, Frost and Elvehjem ('39) have reported that such extracts gave irregular results when fed to rats and suggested that toxic products were generated in the process of acid ether extraction. The findings in this laboratory have been quite different, and we are unable to offer any explanation of this discrepancy. We are agreed that only a part of the activity is extracted by ether or the activity is extracted only very slowly, for after 48 hours' continuous extraction, a considerable activity remains in the aqueous phase (S27). Whether the activity of the two phases is due to the same or different substances is a problem which cannot be settled on the basis of present data. However, a difference in the

[2] A part, but not all, of this difference is explained by the fact that Edgar, El Sadr and Macrae have used only male animals whereas we have used both sexes.

properties of the active material in the ether extract from that of the original solution (S13) is indicated by the different stability to acid hydrolysis. These findings are especially significant in the light of reports from other laboratories (Schultz, '37, '38; Edgar, '37) which indicate a high degree of stability of the active substances of yeast extracts to acid hydrolysis. We are, therefore, led to the tentative hypothesis that the filtrate factor consists of at least one stable and one or more unstable substances.

A clue to the nature of the unstable material is obtained from the method of preparation of S22. The methods used in its preparation are similar to those employed by Snell, Strong and Peterson ('37) and by Williams and co-workers ('38) in the preparation of pantothenic acid, which markedly stimulates the growth of certain yeasts (Williams, '38), lactic acid bacteria (Snell, '38) and the diptheria bacillus (Mueller, '38). Through the kindness of Dr. Leo Rane, our fraction S22 has been tested for its growth-promoting activity on the diphtheria bacillus. This fraction could not contain a significant amount of free β-alanine, because the calcium salt of this amino acid is insoluble in alcohol. Moreover, this fraction was found to contain only a slight trace of amino nitrogen by the nitrous acid method. It was found that the preparation S22 could replace β-alanine in the synthetic medium, a property characteristic of pantothenic acid. This suggested that pantothenic acid might be the active substance of the S22 preparation.

In order to test this hypothesis, a sample of the calcium salt preparation was purified by the procedures of Williams ('38). These procedures involve alcohol precipitation, treatment with mercuric chloride in alcoholic and aqueous solution, and fractionation of an alcoholic solution of the purified calcium salt, by means of isopropyl ether precipitation. This purified material has been found to stimulate growth when administered in doses between 6 and 12 mg. weekly to each animal. It is not yet known whether or not smaller amounts may be equally effective. It appears likely, therefore, that

pantothenic acid is one of the substances necessary for rat growth.

There is not necessarily any discrepancy between this conclusion and the recent identification of pantothenic acid with the chick antidermatitis factor by Jukes ('39) and by Woolley, Waisman and Elvehjem ('39). Oleson, Bird, Elvehjem and Hart ('39) found that concentrates which were active in the prevention of chick dermatitis gave little improvement in the growth of rats. They found, however, that such concentrates brought about a rapid cure of the 'spectacled eye' condition.

Experience has shown that the growth-promoting ability of a given substance depends upon the basal dietary regimen—whether it is or is not the limiting factor. It is entirely possible, therefore, that a substance which simply cures a deficiency symptom in animals on one basal diet could actively stimulate growth in animals on a different basal diet. For these reasons, we believe that pantothenic acid is the active substance with which we are dealing in the calcium salt preparations.

The authors wish to express their gratitude to Dr. Guy W. Clarke of Lederle Laboratories, Inc., for his generosity in supplying the liver extracts used in these experiments, and to Dr. Merton C. Lockhart for assistance in large scale preparations.

SUMMARY

Albino rats grew only slowly when given a basal diet essentially free from B-vitamins, but supplemented by adequate amounts of pure thiamin and riboflavin and by fuller's earth adsorbate from liver extracts. The growth-promoting substances of the fuller's earth filtrate could be concentrated by extraction with amyl alcohol, adsorption and elution from charcoal and by continuous ether extraction of the acid aqueous solution. The activity of the ether extract could be concentrated further by fractionation of the brucine and calcium salts.

It is suggested that pantothenic acid is responsible for part of the growth-promoting activity of liver extracts.

LITERATURE CITED

COHN, E. J., G. R. MINOT, J. F. FULTON, H. F. ULRICHS, F. C. SARGENT, J. H. WEARE AND W. P. MURPHY 1927 The nature of the material in liver effective in pernicious anemia. I. J. Biol. Chem., vol. 74, p. lxix.

EDGAR, C. E., AND T. F. MACRAE 1937 The water-soluble B-vitamins. VIII. Essential dietary factors for the rat present in autoclaved yeast extracts in addition to lactoflavin. Biochem. J., vol. 31, p. 886.

EDGAR, C. E., M. M. EL SADR AND T. F. MACRAE 1938 a The water-soluble B-vitamins. XII. The preparation from liver of fractions containing the same dietary essential factors for the rat as the yeast eluate fraction and the yeast filtrate fraction. Biochem. J., vol. 32, p. 2225.

———— 1938 b The water-soluble B-vitamins. XI. The estimation of yeast eluate factor and yeast filtrate factor by rat growth methods. Biochem. J., vol. 32, p. 2200.

FROST, D. V., AND C. A. ELVEHJEM 1937 Further studies on factor W. J. Biol. Chem., vol. 121, p. 255.

———— 1939 Factor W and its relation to the vitamin B complex. J. Biol. Chem., vol. 128, p. 23.

JUKES, T. H. 1939 Pantothenic acid and the filtrate (chick antidermatitis) factor. J. Am. Chem. Soc., vol. 61, p. 975.

LEPKOVSKY, S., T. H. JUKES AND M. E. KRAUSE 1936 The multiple nature of the third factor of the vitamin B complex. J. Biol. Chem., vol. 115, p. 557.

MUELLER, J. H., AND A. W. KLOTZ 1938 Pantothenic acid as a growth factor for the diphtheria bacillus. J. Am. Chem. Soc., vol. 60, p. 3086.

OLSON, J. J., H. R. BIRD, C. A. ELVEHJEM AND E. B. HART 1939 Additional nutritional factors required by the rat. J. Biol. Chem., vol. 127, p. 23.

OSBORNE, T. B., AND L. B. MENDEL 1919 The nutritive value of the wheat kernel and its milling products. J. Biol. Chem., vol. 37, p. 572.

SCHULTZ, H. W., AND H. A. MATTILL 1937–1938 Studies on the vitamin B complex. J. Biol. Chem., vol. 122, p. 183.

SNELL, E. E., F. M. STRONG AND W. H. PETERSON 1937 Growth factors for bacteria. VI. Fractionation and properties of an accessory factor for lactic acid bacteria. Biochem. J., vol. 31, p. 1789.

———— 1938 Pantothenic and nicotinic acids as growth factors for lactic acid bacteria. J. Am. Chem. Soc., vol. 60, p. 2825.

WILLIAMS, R. J., J. H. TRUESDAIL, H. H. WEINSTOCK, JR., E. ROHRMANN, C. M. LYMAN AND C. H. MCBURNEY 1938 Pantothenic acid II. Its concentration and purification from liver. J. Am. Chem. Soc., vol. 60, p. 2719.

WOOLLEY, D. W., H. A. WAISMAN, O. MICKELSEN AND C. A. ELVEHJEM 1938 Some observations on the chick antidermatitis factor. J. Biol. Chem., vol. 125, p. 715.

WOOLLEY, D. W., H. A. WAISMAN AND C. A. ELVEHJEM 1939 Nature and partial synthesis of the chick antidermatitis factor. J. Am. Chem. Soc., vol. 61, p. 977.

THE EFFECT OF PHOSPHORUS ON THE BIOLOGICAL ESTIMATION OF VITAMIN D ACTIVITY [1]

BRIAN O'BRIEN AND KENNETH MORGAREIDGE

The Institute of Optics and the Department of Biochemistry, The University of Rochester, Rochester

TWO FIGURES

(Received for publication April 25, 1939)

In a previous publication (Morgareidge and O'Brien, '38), we have shown that the addition of vitamin D concentrates to skimmed milk results in an apparent total antirachitic activity for the mixture which is two to three times greater than can be accounted for on the basis of the concentrate added. In the light of experiments reported herewith, it becomes evident that milk phosphorus is the component which plays the dominant role in producing this enhancement effect. Furthermore, the data serve to reemphasize the fact that vitamin D content and total antirachitic potency cannot be used synonymously in any but the most strictly limited cases where supplementary factors are known to be absent. This necessary limitation is inherent in the standard curative assay procedure employing rachitic rats as the test animals.

EXPERIMENTAL

In order to maintain experimental conditions comparable to those employed for the assay of milk samples, it was desirable to feed the vitamin together with a source of phosphorus dispersed in an aqueous protein-containing medium. Five per cent solution of gelatin was found to satisfy the requirements since it provided a medium of very low phosphorus content

[1] Read before the thirty-third annual meeting of the American Society of Biological Chemists at Toronto, April 27, 1939.

(0.0015 mg. per cubic centimeter) in which vitamin D could be stably dispersed by adding a prophylene glycol solution of crystalline D_2. We have found that prophylene glycol solutions of vitamin D cannot be diluted with water alone but that the presence of some protective colloid (such as gelatin or milk protein) is necessary in order to produce stable dispersions of suitable potency for assay feeding.

The essential features of the assay technic may be briefly described as follows: Young rats from our standardized D-assay colony are weaned directly to the 2965 diet of Steenbock and Black ('25). A 21-day depletion period is allowed for the development of rickets. Prior to test-feeding, each animal is examined by radiograph of the left knee-joint and any failing to show uniformly satisfactory rickets are discarded. At the beginning of the test the rats average 40 days of age and 55 to 60 gm. in weight. The test-feeding is carried on for 8 consecutive days and final examination for degree of healing is made on the tenth day by the radiographic technic (O'Brien and Morgareidge, '38). Our standard test-group consists of seven animals and for every series of comparisons, four or five such groups are given graded levels of International Standard vitamin D solution diluted in corn oil and administered from calibrated dropping pipettes. The levels of standard fed are chosen to cover the range of 0.35 to 0.80 I.U. per day in steps corresponding to the ratio of $\sqrt{2}:1$. Differences in vitamin intake of this magnitude are easily distinguished and the response of our colony is such that this range of levels covers the most critical region of the healing curve. The material to be tested is fed simultaneously to four or five groups of rats in such amounts that the resulting healing matches that produced by the standard at at least two levels. If the approximate potency of the material is not known, a preliminary assay is run to establish the proper feeding levels.

In all the experiments reported here, the source of vitamin D was a single bottle of Drisdol (Winthrop Chemical Co.) which is crystalline D_2 dissolved in propylene glycol. Five

per cent solutions of BACTO gelatin (Difco Laboratories) were fortified with this material so that the necessary daily dose was contained in 4.0 cc. of gelatin solution. Using a total of ninety-eight rats in six independent assays, the potency of this sample was determined to be 6.7 I.U. per mg.[2] (average deviation, ±1.4) when fed dispersed with gelatin. The average number of units of healing observed plotted against the number of units fed per day are shown by the solid circles in figure 1. The dotted line is theoretical for a one-to-one correspondence.

In our previous work with vitamin D milks, the dilutions were always so arranged that the daily volume of milk fed was 4.0 cc. The average total phosphorus content of these samples was determined according to the method of Holtz ('29) through the cooperation of Mr. David Alling and found to be 1.0 mg. per cubic centimeter (±0.3 mg.). Therefore, we prepared 5% gelatin solutions containing sodium glycerophosphate calculated to contain 4.0 mg. of phosphorus in 4.0 cc. (based on actual analysis of the glycerophosphate). These phosphate-gelatin solutions were then fortified with varying amounts of vitamin D and the apparent potency of the Drisdol used was calculated from a series of three independent assays employing a total of 105 rats (exclusive of the standard animals). The value obtained was 22.7 I.U. per milligram (average deviation, ±3.4). When the actual amounts of vitamin D fed are compared with the observed healing levels in International Units, the open circles in figure 1 are obtained. Control experiments were run in which the gelatin solution alone and gelatin plus 8.0 mg. of phosphorus per day gave no healing. Thus, when 4.0 mg. of phosphorus were fed daily together with vitamin D, the apparent potency of the vitamin preparation was increased from 6.7 to 22.7 I.U. per milligram, giving an enhancement factor of 3.4 (which is numerically equivalent to the slope of the line

[2] Due to the fact that propylene glycol is very hygroscopic, the absolute potency will decrease with use if the sample is not carefully protected from atmospheric moisture.

drawn through the points obtained at the various healing levels).

Similar experiments were done to determine the effects of 2 mg. and of 1 mg. of phosphorus per day (one experiment each) and the results are shown by the barred and the half-solid circles, respectively, in figure 1. The slopes of these

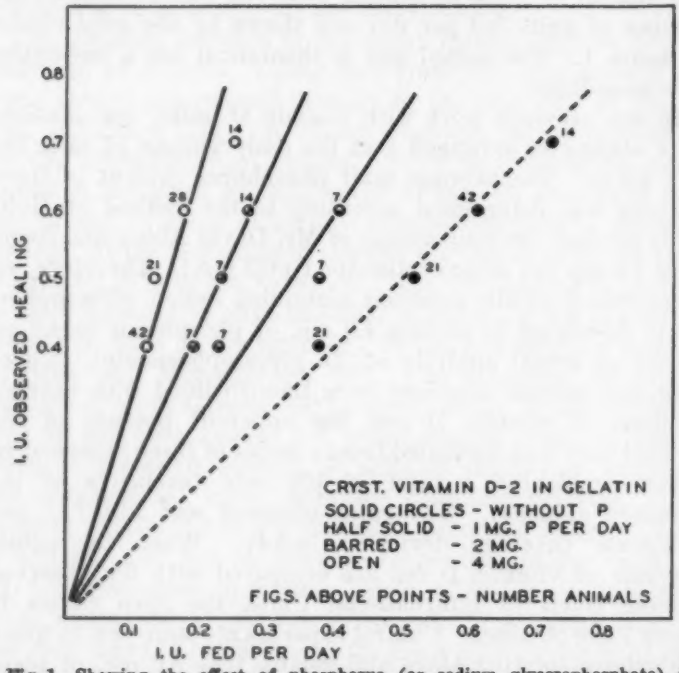


Fig. 1 Showing the effect of phosphorus (as sodium glycerophosphate) fed together with vitamin D_2 on the healing of rachitic rats.

lines can be designated as the 'enhancement factors' for their respective amounts of phosphorus (as sodium glycerophosphate) on the activity of vitamin D. When plotted as shown in figure 2, they are seen to fall on a straight line which is expressed by the equation:

$$E.F. = 1.0 + 0.58\ P \qquad (1)$$

Since the total antirachitic activity of a mixture also depends on the amount of vitamin D present:

$$A = (1.0 + 0.58\,P)\,D \qquad (2)$$

where A is the observed vitamin D activity, P the number of milligrams of phosphorus, and D the number of units of vitamin D.

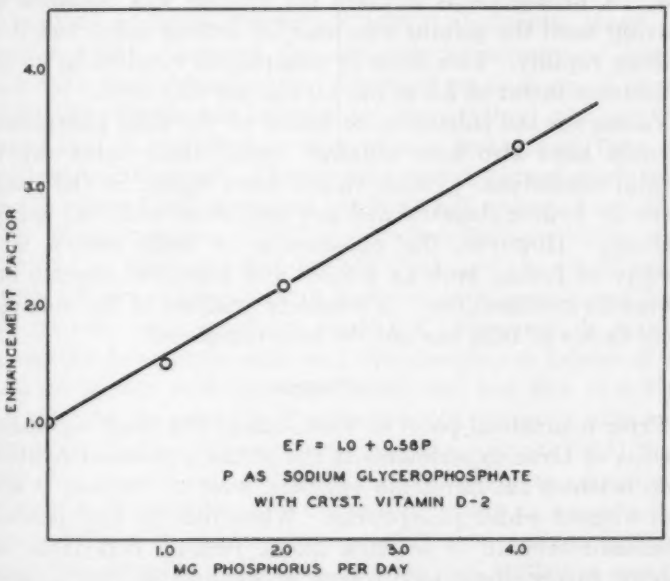


Fig. 2 Showing the relationship between the amount of phosphorus fed and the enhancement of vitamin D activity. In this chart, the points represent the slopes of the lines in figure 1.

We have also conducted some experiments to show that all types of phosphorus containing compounds do not yield the same enhancement factor. Both soluble and insoluble inorganic phosphates have been tested. Primary sodium phosphate (NaH_2PO_4) fed at a level equivalent to 4.0 mg. of phosphorus per day gives a factor of 3.5 (not significantly different from glycerophosphate). The insoluble phosphate tested was hydroxylapatite which was prepared by dissolving

calcium chloride in 5% gelatin solution and then adding phophoric acid to give a caluculated concentration of phosphorus of 1.0 mg. per cubic centimeter. The pH was maintained between 6.0 and 7.0 (Beckman glass electrode) during the addition of the H_3PO_4 by the simultaneous addition of sodium hydroxide. The finely divided amorphous precipitate of $Ca_{10}(PO_4)_6(OH)_2$ settled only slowly in the gelatin solution. A homogeneous mixture for feeding was obtained by stirring until the gelatin was near its setting point and then chilling rapidly. This form of phosphorus resulted in an enhancement factor of 2.2 at the 4.0 mg. per day level.

Values for the enhancement factor of the total phosphorus of milk have also been obtained under these same experimental conditions. These values have fallen in the range given by hydroxylapatite and are consistent with our earlier findings. However, the phosphorus of milk occurs in a variety of forms, both as soluble and insoluble organic and inorganic combinations. A complete analysis of the enhancement factor of milk has not yet been completed.

DISCUSSION

From a practical point of view, one of the most significant results of these experiments is the actual numerical relationship between the minimum effective level of vitamin D with and without added phosphorus. When fed the International Standard vitamin D solution alone, rachitic rats from our colony rarely show any traces of healing at levels below 0.35 I.U. per day for 8 days. This minimum level varies from colony to colony and from time to time in the same colony. For assay purposes we usually find 0.40 to 0.45 I.U. per day to represent the lowest practical dose. The fact that an intake of 4.0 mg. of phosphorus per day in addition to that obtained from the basal diet lowers this minimum effective level of vitamin D intake to the neighborhood of 0.1 I.U. is of importance. This is especially true when one considers that, to initiate healing, the rat requires twice this amount of phosphorus and nearly four times this much vitamin D when either is fed alone.

The problem of the bio-assay of vitamin D milks is of great interest to many. Obviously, it becomes necessary, in defining the antirachitic potency of milk, to recognize that it is determined by the product of the phosphorus by the vitamin D content. It is interesting to note in this connection that human milk contains only about one-fifth to one-sixth as much phosphorus as cow's milk. It seems reasonable that this fact may largely account for the very low apparent vitamin D content reported for breast milk. Outhouse, Macy and Brekke ('28) compared the antirachitic potency of human milk with that of cow's milk. They fed up to 40 cc. of human milk (the volume was reduced by evaporation) per day and found that it produced no appreciable increase in bone ash over rachitic controls. Thirty cubic centimeters of cow's milk, on the other hand increased the bone ash values from 31 to 41% (a net increase of 10%). The rachitogenic ration used was that of Osborne, Mendel and Park and the Ca/P ratios of the dietary intakes during both the rachitogenic and healing periods were carefully equalized. However, it may be calculated from their data that the phosphorus intake of the rats on human milk was nearly 8.0 mg. per day (Ca/P = 4–5:1). At the same Ca/P ratio, the rats on cow's milk were receiving 19 mg. of phosphorus per day. Shohl ('36), however, has clearly demonstrated that the absolute amounts of calcium and phosphorus in the diet are fully as important in characterizing its rachitogenic properties as are the ratios of these two elements. His data indicate that Outhouse and her associates used a diet in assaying human milk which was considerably more rachitogenic that that used for cow's milk.

Finally, it is necessary to point out that the rachitic rat is in a state of acute phosphorus starvation on the high-calcium, low-phosphorus diets commonly used in assay work. That this dietary ratio of calcium to phosphorus is of foremost importance in the production of rickets has long been recognized. It is not surprising, therefore, to find that even a partial restoration toward normal of the intake of these two elements has a profound influence on the efficiency with which vitamin D promotes the healing processes. At the

present time there is no experimental basis for assuming that the findings reported here with regard to vitamin D and phosphorus apply to any species other than the rat or to any other set of experimental conditions than those described.

SUMMARY

It has been found under conditions commonly employed for the biological estimations of vitamin D that phosphorus added to the vitamin supplement greatly enhances its apparent potency. The equivalent healing is found to be proportional to the product of the vitamin by the phosphrous fed. For example, 4.0 mg. of phosphorus per day for 8 days (as sodium glycerophosphate) enhances the potency of crystalline vitamin D_2 by a factor of 3.4 times. All phosphorus containing compounds do not possess the same enhancement factor. The significance of this relationship in the estimation of the antirachitic potency of phosphorus containing foods (such as milk) is pointed out.

ACKNOWLEDGMENT

It is a pleasure to acknowledge the financial assistance of the Wisconsin Alumni Research Foundation of Madison, Wisconsin, in carrying out this work. We are indebted to Mr. David Alling for numerous phosphorus determinations.

LITERATURE CITED

HOLTZ, FRIEDRICH 1929 Micro-Phosphatbestimmung. Biochem. Z., Bd. 210, S. 252.

MORGAREIDGE, KENNETH, AND BRIAN O'BRIEN 1938 Anomalies in the bioassay of vitamin D milk. J. Nutrition, vol. 16, p. 395.

O'BRIEN, BRIAN, AND KENNETH MORGAREIDGE 1938 Radiographic demonstration of protection by vitamin D against metaphyseal decalcification in adult rats on high-calcium, low-phosphorus diet. J. Nutrition, vol. 16, p. 91.

OUTHOUSE, JULIA, ICIE G. MACY AND VIOLA BREKKE 1928 Human milk studies. A quantitative comparison of the antirachitic factor in human milk and cow's milk. J. Biol. Chem., vol. 78, p. 129.

SHOHL, A. T. 1936 Rickets in rats. The effect of low-calcium, high-phosphorus diets at various levels and ratios upon the production of rickets and tetany. J. Nutrition, vol. 11, p. 275.

STEENBOCK, H., AND A. BLACK 1925 Fat soluble vitamins. XXIII. The induction of growth promoting and calcifying properties in fats and their unsaponifiable constituents by exposure to light. J. Biol. Chem., vol. 64, p. 263.

SOYBEAN HAY, ALFALFA HAY AND YELLOW CORN AS VITAMIN A SUPPLEMENTS IN COTTONSEED MEAL RATIONS FOR GROWING CATTLE [1]

J. O. HALVERSON, EARL H. HOSTETLER, J. E. FOSTER AND F. W. SHERWOOD

Divisions of Animal Nutrition and Animal Husbandry, North Carolina Agricultural Experiment Station, Raleigh

(Received for publication May 4, 1939)

In a prior publication from this station (Halverson and Sherwood, '30) it was shown that the so-called cottonseed meal injury to cattle is due primarily to a lack of vitamin A in rations containing comparatively large amounts of cottonseed meal but without a leafy roughage. The investigation described here was begun in 1928 in order to confirm the earlier conclusion and to determine the amount of yellow corn, alfalfa hay or soybean hay required to prevent deficiency symptoms and to promote normal growth in young cattle, and also to determine the minimum daily carotene requirement for growth.

The syndrome of vitamin A avitaminosis is now well known (Jones et al., '26; Bechdel et al., '28; Kuhlman et al., '30–'32; Hart and Guilbert, '33). Several investigators have produced these symptoms in cattle by feeding cottonseed meal supplemented with feeds which contain little or no vitamin A. They have also been able to prevent or cure the condition by the use of good quality hay or other vitamin A carriers (Reed et al., '28; Bechdel, '31; Bechdel and Williams, '32; Copeland and Fraps, '32; Hart and Guilbert, '33; Kuhlman et al., '34–'36, and Bechdel and Skaggs, '35).

[1] Published with the approval of the acting director of the North Carolina Agricultural Experiment Station as no. 105 of the journal series.

Fraps and Treichler ('33), Russell et al. ('34) and Wiseman et al. ('38) have found that the vitamin A activity (carotene) in alfalfa hay is quite variable depending upon the grade or quality of the hay, the method of curing and the time in storage. Wiseman et al. ('38) state that the carotene content of U. S. no. 1 alfalfa hay varies between 19 and 121 mg. per kilogram. The no. 2 grade contains 12 to 20 and the no. 3 grade carries 1 to 11 mg. per kilogram.

Hilton et al. ('35) reported that field-cured young alfalfa hay had a vitamin A activity equivalent to 18 Sherman-Munsell units per gram and that their late alfalfa hay contained 20 units. They also found that young and late soybean hays respectively contained the equivalent of 36 and 8 Sherman-Munsell vitamin A units per gram.

Fraps ('31) found twenty samples of yellow corn to contain 2.5 to 8 Sherman-Munsell units vitamin A activity per gram. Wiseman et al. ('38) say that yellow corn contains 3 to 9 mg. carotene per kilogram, but they say that these figures include other pigments which go into the carotene fraction.

Guilbert and Hart ('35) found that the daily minimum carotene requirement of the bovine is 26 to 33 μg. per kilogram live weight when the carotene is supplied in alfalfa hay. A carotene intake at the level of 29 μg. per kilogram daily prevented or cured clinical symptoms and promoted normal weight increase, yet it resulted in no storage. When the intake fell below this level, night blindness reappeared and gains decreased. They also advanced the hypothesis that the vitamin A requirement is related to body weight rather than to energy requirement.

Ward et al. ('38) found that when alfalfa hay or carotene in cottonseed oil was used as the source of vitamin A, 12 to 14 μg. of carotene per pound of body weight per day was sufficient to prevent the symptoms of vitamin A deficiency in growing calves. Increased growth or improved well being in Holstein calves did not result when the carotene was supplied above 20 to 30 μg. per day per pound body weight. Work

with older animals indicates that heifers 1 to 2 years of age will probably show little or no carotene deficiency in the winter months if they have been on good pasture during the previous summer.

The work of Meigs and Converse ('38) indicates that the carotene requirement for successful reproduction is considerably higher than the minimum for growth or the prevention of night blindness.

EXPERIMENTAL

A series of five consecutive feeding trials or experiments was conducted. In the first experiment grade Shorthorn steers were used. High grade Shorthorn heifers were used in experiments II, III and IV. In the last experiment there were four Hereford steers, nos. 2, 3, 6 and 7; one Angus steer no. 5; two heifers, nos. 1 and 4 which were Hereford and Angus crosses and one Hereford and Shorthorn cross bred heifer, no. 8. The animals in all experiments were taken off of grass pasture in the late autumn and all except those in experiment V were given a ration of shelled white corn and soybean hay at a little above the maintenance level until placed on the experimental rations. The animals in all experiments except no. 1 were spring calves 7 to 9 months old. In experiment I they were approximately 20 months old when taken from pasture. They were put in the experiment 88 days later. The lengths of the preliminary feeding period in the subsequent experiments were 7, 33, 36 and 0 days respectively.

All of the animals were individually stall fed, but during the day they were allowed to run together in a paddock from which all grass, weeds and other feeds were carefully excluded. Daily records were made of the feed consumed and the animals were weighed fortnightly.

For better control in feeding, the concentrates and roughages were not fed separately but were ground and thoroughly mixed, enough of the ration to last approximately 3 weeks

being prepared at one time. The composition of the rations used is shown in table 1.

The alfalfa meal had a bright green color and was of excellent quality. While the alfalfa hay graded no. 3 it varied somewhat in quality for each experiment. This hay was locally grown and was mixed with fox tail (Chaetochloa glauca) and crab grass (Syntherisma sanguinaly). The soybean hay was of good quality and usually had a bright green

TABLE 1

Percentage composition of rations used

Ration no.	1	2[1]	3	4	5	6	7	8	9
Cottonseed meal (36% protein)	48	49.0	24.5	44.7	26	26	26	26	8.2
Cottonseed hulls	25	24.5	24.5	22.3	22	27	27	17	—
Beet pulp	25	24.5	24.5	22.3	25	—	—	—	—
Yellow corn (no. 2 or 3)	—	—	24.5	—	26	—	—	—	—
White corn	—	—	—	—	—	26	26	26	59.4
Alfalfa meal	—	—	—	8.9	—	—	—	—	—
Alfalfa hay	—	—	—	—	—	20	—	—	—
Soybean hay	—	—	—	—	—	—	20	30	32.4
Mineral mixture[2]	2	2.0	2.0	1.8	1	1	1	1	—

[1] Fed with 0.99 and 0.97 ounce cod liver oil daily 168 days to animals nos. 11 and 12 respectively; increased to 1.99 ounces daily for the next 226 and 227 days.

[2] Equal parts of calcitic limestone and steamed bone meal, common salt being always available.

color. Both kinds of hay were baled and stored in a rather dark barn loft.

In the last three experiments both the hay and the corn were sampled when first received. Later other samples of the hays were taken when they were being ground. These samples were kept in duplicate well filled sealed glass jars, in a refrigerator and dairy cooler until assayed for vitamin A by the rat growth method. International standard carotene was used as the reference standard in the assay. The results are given in table 2.

In computing the vitamin A intake of the animals in experiments I and II the following average values, in international units per gram, were used: yellow corn 2.5, alfalfa meal 11, alfalfa hay 7, soybean hay 19 and cod liver oil 600. The cod liver oil used was a grade sold for animal feeding and guaranteed to contain not less than 600 international vitamin A units per gram.

The earlier symptoms regarded as definite indications of avitaminosis A were a pronounced loss of appetite, a rough unkempt hair coat and listlessness. The eyes also were often affected at this stage. They became rather dry with a dull glassy appearance, followed by lacrymation and nyctalopia.

TABLE 2

Vitamin A activity of white corn, yellow corn, alfalfa hay and soybean hay used in experiments III, IV and V in international units of vitamin A per gram

Experiment	III	IV		V				
Month of exp. when sample taken	Initial	Initial	4th	Initial	3rd	5th	11th	14th
White corn	—	<0.5	—	0	—	—	—	—
Yellow corn	2.5	1.5	—	2.5	—	—	—	—
Alfalfa hay	10.0	3.0	5	11.0	—	6	5	7[1]
Soybean hay	22	10.0	20	17.0	14	22	22	19

[1] Composite of samples taken thirteenth to fifteenth months.

RESULTS

The results of the experiments are presented according to similarities in the vitamin A intake and the behavior of the animals.

Table 3 lists the data pertinent to the animals receiving the basal cottonseed meal ration no. 1, the rations containing yellow corn, nos. 3 and 5, ration 4 containing 8.9% alfalfa meal and ration 6 with 20% alfalfa hay.

It is probable that a part of the vitamin A reserves of the rather large steers in experiment I was used in the 88-day preliminary period in which they received a limited amount of shelled white corn and soybean hay. As a consequence they developed incipient symptoms of vitamin A avitaminosis

TABLE 3

Rations with a low vitamin A content

Ration Vitamin A supplement	Experiment	Animal	Vitamin A intake per kilo body weight per day		Body weight			Average daily gain		Length of period		Deficiency symptoms at end
			Fore-period[1]	Entire period	Initial	End of fore-period	Final	Fore-period	Entire period	Fore-period	Entire period	
No.		No.	No.	I.U.	I.U.	lbs.	lbs.	lbs.	lbs.	lbs.	days	days	
1	None	I	1	0	0	835	1205	1025	2.64	1.01	140	187	Severe
1	None	I	2	0	0	823	1005	865	1.62	0.20	112	210	Severe
1	None	I	3	0	0	825	975	650	1.34	—	162	167	Died
1	None	I	4	0	0	745	930	720	1.65	—	112	165	Died
1	None	I	5	0	0	835	1035	940	1.79	0.56	112	186	Died
1	None	II	7	0	0	395	715	655	1.63	1.03	196	252	Severe
1	None	II	8	0	0	275	530	505	1.52	1.10	168	210	Severe
3	24.5% yellow corn	II	9	16	15	370	685	690	1.89	1.03	168	196	Severe
3	24.5% yellow corn	II	10	16	15	280	550	522	1.29	1.07	210	226	Severe
5	26.0% yellow corn	IV	1	11	9	327	625	575	1.93	1.11	154	224	Severe
5	26.0% yellow corn	IV	2	12	11	318	640	610	2.09	1.68	154	174	Severe
5	26.0% yellow corn	V	1	—	11	475	—	630	—	0.69	—	234	Moderate
5	26.0% yellow corn	V	2	15	14	310	475	500	1.27	1.07	130	178	Severe
4	8.9% alfalfa meal	II	13	—	23	320	—	1000	—	1.73	—	394	None
4	8.9% alfalfa meal	II	14	23	22	315	915	897	1.43	1.06	420	453	Severe
6	20.0% alfalfa hay	IV	3	21	19	378	980	985	1.78	1.64	338	371	Incipient
6	20.0% alfalfa hay	IV	4	25	28	245	675	680	1.71	1.35	252	323	Severe
6	20.0% alfalfa hay	V	3	—	28	465	—	975	—	1.19	—	430	None
6	20.0% alfalfa hay	V	4	—	33	325	—	535	—	0.66	—	318	Moderate

[1] The fore-period is that part of the experimental period in which the animal was apparently normal before symptoms of vitamin A deficiency became manifest.

in a shorter time than the smaller heifers in experiment II, which were put on the experimental basal ration 1 week after removal from grass pasture.

The time required for symptoms to develop in the six head fed approximately 25% yellow corn in their ration did not differ significantly from that of the comparable negative controls (nos. 7 and 8, experiment II) even though they got 9 to 15 international units of vitamin A per kilogram body weight daily. When 25% of yellow corn is the sole source of vitamin A in a cottonseed meal ration it does not supply enough of this essential to protect young cattle or even to postpone materially the time of nutritive failure.

Four of the six head fed 8.9% alfalfa meal or 20% no. 3 alfalfa hay exhibited symptoms of vitamin A deficiency in 252 to 420 days and two survived 394 and 430 days without failure. Since the vitamin A intake of these animals was 19 to 33 units per kilogram per day, alfalfa hay or meal supplying these amounts of vitamin A delays the time of failure but does not offer safe protection after 8 months.

None of the twelve head in table 4 fed the cottonseed meal ration supplemented with approximately 1.5 ounces of cod liver oil per day or with 20% or more of soybean hay exhibited any symptoms of vitamin A deficiency in over a year of the experimental regime. The least amount of vitamin A ingested by any of these animals averaged 59 international units daily per kilogram of body weight. At this level of vitamin A intake the average daily gains were somewhat greater than those listed in table 3 for the entire period although the animals receiving sub-minimal amounts of vitamin A were able to make satisfactory gains during the earlier part of the experiment before their reserves had been exhausted.

The results shown in tables 3 and 4 are in excellent agreement with those of Guilbert and Hart ('35) and of Ward et al. ('38). The former found the minimum daily carotene (in alfalfa hay) requirement to be equivalent to 43 to 55 international vitamin A units per kilogram body weight and the

TABLE 4

Rations with a high vitamin A content

Ration no.	2		7				8				9	
Vitamin A supplement	Cod liver oil		20% soybean hay				30% soybean hay				32.4% soybean hay	
Experiment no.	II		IV		V		IV		V		II	
Animal no.	11	12	5	6	5	6	7	8	7	8	8a[1]	15
Vitamin A intake, I.U. per kilogram live weight per day	81	90	59	60	82	89	87	85	110	138	162	156
Initial weight, lbs.	350	280	328	308	450	345	352	275	445	405	207	310
Final weight, lbs.	950	960	1055	940	1052	980	1035	868	1038	740	595	1000
Gain, lbs.	600	680	727	632	602	635	683	593	593	335	388	690
Average daily gain, lbs.	1.52	1.56	1.95	1.67	1.40	1.45	1.83	1.57	1.39	0.64[2]	1.33	1.58
Days fed	394	435	373	378	432	439	373	378	437	521	297	436

[1] 8a was a calf of no. 8 experiment V. The record presented here starts at the time the calf reached the age of 171 days although since birth it had not received any feed except its mother's milk and ration no. 8.

[2] This heifer was stunted because of a gestation and lactation period.

values of the latter are equivalent to 44 to 52 units per kilogram for carotene in oil.

The beef animals receiving 20% soybean hay in their ration reached a practical slaughter weight with a satisfactory degree of finish in 12 to 14 months without any evidence of vitamin A deficiency. So far as rate of gain and physical well being were concerned 30% soybean hay did not yield results that were superior to those obtained when 20% was fed although the estimated daily vitamin A intake per kilogram body weight was considerably below the five to ten times the minimal for the prevention of night blindness which Guilbert et al. ('37) recommended as a desirable minimum for practical purposes. The results from the one animal on ration 9 containing 32.4% soybean hay and from the two whose basal ration was supplemented with cod liver oil were very similar to those from the animals given 20 or 30% soybean hay.

These observations indicate that the minimum for practical purposes proposed by Guilbert and co-workers may be too high if only the immediate needs of the animal are considered. On the other hand, it is quite possible that a larger vitamin A intake is necessary, especially for lactating cows, to insure maximum storage in the body and a high vitamin A activity in the milk.

Heifer 8, experiment V, was pregnant when placed on the experiment, although only about 10 months of age. On the two hundred and forty-ninth day of the experiment she gave birth to a normal calf which she nursed successfully while continuing on the cottonseed meal ration containing 30% soybean hay. During the gestation period her daily vitamin A intake averaged 109 international units per kilogram of body weight, but because of a proportionately greater appetite in the lactation period she averaged 138 units per kilogram per day for the entire feeding trial. Neither the dam nor her calf, which was continued on the experimental ration, showed any signs of vitamin A deficiency.

The details for the eight animals in experiment III are not given because the experiment was terminated by fire at 140

days. In this trial, which was essentially the same as experiments IV and V, no evidence of vitamin A deficiency was manifest although two head received only 18 units of vitamin A per kilogram per day with a daily gain of 2.09 pounds compared to 1.92 for the other six head. This result is further evidence that weaned calves may be safely carried at least 4 months on rations containing large amounts of cottonseed meal and inadequate amounts of vitamin A when they have previously been on summer pasture.

Of the nineteen animals listed in table 3 three died, two did not develop deficiency symptoms and were slaughtered and four received therapy treatments the results of which are not pertinent to the present discussion. The rations of the remaining ten head were modified by the addition of more vitamin A by feeding cod liver oil to four head, alfalfa hay to two and soybean hay to four head. Increasing the alfalfa hay in ration 6 from 20 to 30% or the substitution of 10 or 20% soy bean hay for an equal amount of cottonseed hulls in ration 5 converted these inadequate diets into rations which cured the symptoms of vitamin A deficiency and enabled the cattle to make satisfactory gains in weight.

CONCLUSIONS

A ration for cattle composed largely of cottonseed meal and cottonseed hulls is deficient in vitamin A precursors. Because of its low vitamin A activity there is no practical level at which yellow corn may be incorporated in a cottonseed meal-cottonseed hulls ration as an adequate vitamin A supplement. No. 3 alfalfa hay is a rather poor source of carotene. When incorporated in the basal ration at a level of 20% of the entire ration it does not supply the minimum vitamin A needs of young growing cattle. Probably 50 to 60% of no. 3 alfalfa hay in the ration will furnish more than the minimum requirement for vitamin A precursors.

The soybean hay used in these experiments had approximately twice the vitamin A activity of the alfalfa hay.

Rations containing 20% soybean hay were adequate for growth and the physical well being of young beef cattle. When 30% was present in the ration one heifer had a successful gestation and lactation period.

These results are in accord with the findings of Guilbert and Hart ('35) and of Ward et al. ('38) that the minimum daily carotene requirement of cattle is equivalent to approximately 43 to 55 international vitamin A units per kilogram of body weight.

In the cotton belt where these feeds are comparatively cheap a ration composed of cottonseed meal, cottonseed hulls and a good hay in sufficient quantity is a practical one for growing cattle.

LITERATURE CITED

BECHDEL, S. I., H. E. HONEYWELL AND R. A. DUTCHER 1928 The effect of feeding heifers a ration deficient in vitamin A. Penn. Agr. Exp. Sta., 41st Annual Report, pp. 20–21.

———— 1931 The relation of vitamin A to the nutritive value of cottonseed meal. Penn. Agr. Exp. Sta. 44th Annual Report, Bull. 266, pp. 19–20.

BECHDEL, S. I., AND P. S. WILLIAMS 1932 The nutritive value of cottonseed meal. Penn. Agr. Exp. Sta., 45th Annual Report, Bull. 279, pp. 8–9.

BECHDEL, S. I., AND S. R. SKAGGS 1935 A study of the malnutrition incident to the heavy feeding of cottonseed meal. J. Dairy Sci., vol. 18, abstract, Proc. Am. Dairy Sci. Assoc., pp. 435–436.

COPELAND, O. C., AND G. S. FRAPS 1932 Sorghum silage as a source of vitamin A for dairy cows. Tex. Agr. Exp. Sta. Bull. no. 473, p. 11.

FRAPS, G. S. 1931 Variations in vitamin A and chemical composition of corn. Tex. Agr. Exp. Sta. Bull. no. 422, pp. 44–45.

FRAPS, G. S., AND R. TREICHLER 1933 Vitamin A content of foods and feeds. Tex. Agr. Exp. Sta. Bull. no. 477, p. 29.

GUILBERT, R. F., AND G. H. HART 1935 Minimum vitamin A requirements with particular reference to cattle. J. Nutrition, vol. 10, pp. 409–427.

HALVERSON, J. O., AND F. W. SHERWOOD 1930 Investigations in the feeding of cottonseed meal to cattle. N. C. Agr. Exp. Sta. Tech. Bull. 39, pp. 1–158.

HART, G. H., AND R. F. GUILBERT 1933 Vitamin A deficiency as related to reproduction in range cattle. Calif. Agr. Exp. Sta. Bull., no. 560, pp. 3–30.

HILTON, J. S., S. W. HAUGE AND J. W. WILBUR 1935 The vitamin A activity of butter produced by cows fed alfalfa hay and soybean hay cut in different stages of maturity. J. Dairy Sci., vol. 18, pp. 795–800.

JONES, I. R., C. H. ECKLES AND L. S. PALMER 1926 The role of vitamin A in the nutrition of calves. J. Dairy Sci., vol 9, pp. 119–139.

KUHLMAN, A. H., E. WEAVER AND W. D. GALLUP 1932–1934 The use of cottonseed meal in dairy rations. Applying science to agriculture, Okla. Agr. Exp. Sta. Report, Dairy Problems, pp. 153–166.

———— 1934–1936 Science Serving Agriculture. Okla. Agr. Exp. Sta. Rep., pp. 8–14.

MEIGS, E. B., AND H. T. CONVERSE 1938 The carotene requirements for normal reproduction. J. Dairy Sci., vol. 21, Proc. Am. Dairy Sci. Assoc., abstract, pp. 114–115.

REED, O. E., C. F. HUFFMAN AND L. H. ADDINGTON 1928 Cottonseed meal as a feed for dairy calves. J. Dairy Sci., vol. 11, pp. 488–505.

RUSSELL, WALTER C., M. W. TAYLOR AND D. F. CHICESTER 1934 The effect of the curing process upon the carotene and vitamin A content of alfalfa. N. J. Agr. Exp. Sta. Bull. 560, pp. 1–8.

WARD, R. E., S. I. BECHDEL AND N. B. GUERRANT 1938 The carotene requirement for dairy calves. J. Dairy Sci., vol. 21, abstract, Proc. Am. Dairy Sci. Assoc., pp. 168–169.

WEISMAN, H. G., E. A. KANE, L. A. SHINN AND C. A. CARY 1938 The carotene content of market hays and corn silage. J. Agr. Res., vol. 57, pp. 635–669.

THE FOOD OF THE PRESENT-DAY NAVAJO INDIANS OF NEW MEXICO AND ARIZONA

THORNE M. CARPENTER AND MORRIS STEGGERDA
Nutrition Laboratory, Boston, Massachusetts, and Department of Genetics, Cold Spring Harbor, Long Island, New York, of the Carnegie Institution of Washington

(Received for publication May 4, 1939)

Between 1932 and 1935 a study was made of the food habits of the Maya Indians of Yucatan (Benedict and Steggerda, '36) in which it was found that their food was chiefly carbohydrate (maize). This was challenging, as the basal heat production of the Maya averages 5 to 8% higher than the commonly accepted standards for white men living in the northern part of the United States (Williams and Benedict, '28; Shattuck and Benedict, '31; Steggerda and Benedict, '32). For comparative purposes it was desired to have information regarding the diet and the basal heat production of other Indian groups. Consequently between 1934 and 1936 a study was made of the food habits of the Navajo Indians, chiefly in the southern part of the Navajo reservation in New Mexico and Arizona, but up to the present time a study of the basal heat production of the Navajo has not been feasible. Sixty-six samples of foods in the Navajo's daily diet, both uncooked and cooked, were collected for analysis as to their fat, nitrogen and water content, and their energy value. In addition, data were secured with regard to the character and the amount of food eaten per Navajo family per week.

The Indians who supplied us with food samples and information regarding their food habits were living in hogans on the reservation. They were relatively pure Navajos, although it would be impossible to say with assurance that

any Indian is pure Navajo or to describe the genetic constitution of this group. A Navajo interpreter relayed our questions and in turn secured the information for us.

COMPOSITION AND ENERGY VALUE OF NAVAJO FOODS

The food samples were placed in pint, 'quick seal' metal cans with press-in covers. These were sealed air-tight and shipped to the Nutrition Laboratory in Boston for analysis. No preservative was added to those samples that were dry when collected. Decomposition of the samples that were stored while fresh was guarded against by sprinkling a few grams of benzoic acid over the top of the sample and mixing it with the contents of the can, the food and the acid occasionally being ground in a food chopper to obtain more thorough mixing. In some instances a few drops of formaldehyde were added instead of the benzoic acid. In Boston each sample was ground and placed in an oven at 60°C., after which it was exposed to room air for 24 hours to take on moisture from the atmosphere and was then weighed. This process was carried out a second time, and unless the two air-dry weights thus obtained for each sample (weighed to the nearest 0.01 gm.) agreed within 1%, the drying process was repeated.

The oxy-calorimeter (Benedict and Fox, '25; Benedict, '29) was used to determine the heats of combustion of the air-dry samples. It was assumed that each liter of oxygen consumed during the combustion of the food sample in the oxy-calorimeter represented 4.825 calories. Correction was made for the energy value of the benzoic acid (6.3 calories per gram—Benedict and Fox, '25) in those instances where it was used as a preservative, but no correction was made for the slight loss (10%) of benzoic acid in the air-drying process. The fat content was determined by ether extraction. When benzoic acid had been added to the sample, it was assumed that it was extracted by the ether during the analysis and that none of it escaped in vapor form. The weight of the benzoic acid was, therefore, deducted from the weight of the apparent

total fat in the sample. The nitrogen content was determined by the usual Kjeldahl method. No corrections were made for the formaldehyde used as a preservative. In those instances where sufficient sample was collected, the analyses were made in duplicate, and if the results did not agree within 2% of the total value found for oxygen and 5% of the total values found for nitrogen and fat, further analyses were made until agreement within these limits was obtained.

The results are reported in table 1 on the basis of air-dry material, but the percentage losses in weight of the foods brought from the condition as collected to an air-dry condition are also reported, so that the percentages of nitrogen and fat and the calories per gram can be calculated on the basis of the fresh weight, if desired. The absolute water content of the air-dry material (heated at 100°C.) is likewise reported in those instances where sufficient sample was collected to permit this determination. In some instances, although repeated determinations were made of the same factor, well-agreeing results were not obtained. In other instances only one determination or no determination of a particular factor could be made, owing to insufficient sample. These results are reported, although it is recognized that they have not been corroborated. For comparison with the yellow and the blue Indian corn, analyses were also made of Navajo, Maya, and Illinois white corn.

The fat and the nitrogen content of the different foods varied considerably, as would be expected, but the energy content was in most instances between 3.5 and 4.5 calories per gram of air-dry matter. The meats, which contained considerable fat, and also some of the native berries and seeds had higher energy values. Comparison with analyses of Maya foods (Benedict and Steggerda, '36) is possible in the case of the Maya white corn, the squash and the Chile pepper. This comparison indicates that, although the fat and the nitrogen content of these Maya and Navajo foods differ somewhat, the energy values of the foods are essentially the same.

TABLE 1

Analyses of typical Navajo foods

FOOD	WEIGHT LOST IN DRYING TO AIR-DRY CONDITION [1]	ON AIR-DRY BASIS [2]			
		Moisture [2]	Fat	Nitrogen	Calories per gram
	%	%	%	%	
Dry corn, roasted corn, corn flour					
Indian corn, yellow and blue	4.3	5.5	5.8	1.5	4.3
Navajo white corn	0.8	4.7	5.9	1.4	4.2
Maya white corn	3.0	5.0	4.1	2.0	4.3
Illinois white corn	4.8	3.8	4.3	1.4	4.2
Roasted corn	5.6	2.9	5.7	1.6	4.2
Roasted corn	7.4	2.9	6.0	1.7	4.4
Parched corn	3.9	4.1	5.2	1.8	4.2
Corn flour	0.0	7.9	5.6	1.7	4.1
Breads, dumplings, and mush					
Fried bread	18.7 [4]	9.6 [4]	5.2	2.2	4.1
Baked bread	24.6 [4]	7.7 [4]	1.9	1.9	3.8
Baked blue corn cakes	5.6	3.2	3.8	1.7	4.2
Wheat bread	4.2	4.8	5.1	2.4	4.2
Navajo yeast bread	43.0 [4]	6.7 [4,5]	0.0	4.4	4.1
White bread	6.8	2.5	3.1	2.4	4.4
Green corn tamale bread	...	..	...	1.9	3.9
Green corn tamale bread	4.1	3.7	4.2	1.7	4.2
Ash corn bread	3.0	4.4	4.1 [5]	1.7	4.1
Milk corn cakes	8.1	..	3.3 [5]	1.7 [5]	4.2 [5]
Poured bread	4.2	4.3	3.8	1.7	4.2
Sprouted wheat corn bread	3.7	3.4	5.2	1.8	4.3
Navajo corn cake (alkandt)	4.2	2.2	4.7	1.7	4.4
Green corn bread	4.1	4.3 [5]	3.2	1.8	4.1
Blue paper bread	3.9	4.5	2.8	1.6	4.1
Boiled blue corn cakes (dumplings)	6.8	3.0	3.3	1.7	4.3
Corn mush with cedar ash	63.7 [4]	6.7 [4]	0.05	1.8	4.1
Corn mush and salt	86.4	..	0.0 [6]	1.5	3.9
Vegetables					
Dried squash	0.0	..	1.0 [4]	0.4	3.5
Dried squash	...	..	2.5	0.4	3.6
Pinto beans	3.7	5.2	1.9	3.1	4.1
Wild potatoes and clay [7]	24.9	2.9	0.2	0.7	..
Onion	87.9	..	1.5	1.3	3.6
Wild carrot [8]	...	..	2.5	1.0	..
Chile pepper	0.0	..	...	2.1 [4]	5.3
Wild celery [9]	...	..	...	2.4 [4]	3.4 [4]
Fruits, nuts, and berries					
Dried Yucca	...	..	1.2	0.3	3.5
Dried Yucca	7.3	9.8	1.1	0.3	3.6
Dried peaches	...	6.5	1.4	0.9	3.6
Dried muskmelon	4.2	6.3	1.0	0.6	3.3

TABLE 1 (*continued*)

FOOD	WEIGHT LOST IN DRYING TO AIR-DRY CONDITION [1]	ON AIR-DRY BASIS [2]			
		Moisture [3]	Fat	Nitrogen	Calories per gram
	%	%	%	%	
Fruits, nuts, and berries (continued)					
Dried watermelon	6.3	21.4	1.5	1.4	3.4
Pinon nut butter	0.7 [4]	6.0 [4]	54.4	2.1	6.3
Pinon nut butter	2.8	3.2	24.0	1.7	5.5
Acorns [5]	...	6.7	5.8	1.7	4.1
Red Squaw berries	3.1	..	16.7 [4]	1.7	5.3
Wolf berries [5]	...	12.0	4.4	2.1	4.2
Choke cherry cakes	5.5	3.2 [5]	6.2	0.7	4.4
Ground cherries [5]	...	9.9 [6]	12.0 [6]	2.5 [6]	4.8 [6]
Silver nightshade berries [5]	...	..	...	2.4 [6]	5.0 [6]
Seeds of grasses and weeds					
Thistle seed	0.0	..	1.6	2.2	4.1
Dried seed	0.0	..	7.4 [5]	2.6 [6]	4.3
Weed seed	0.0	..	...	4.1 [6]	6.3 [6]
Sunflower seed [5]	...	..	...	3.2 [6]	5.9 [6]
Tumbleweed seed [5]	...	..	0.1 [6]	2.1 [6]	3.9 [6]
Sage seed [5]	...	..	0.3 [5]	2.8 [6]	4.2 [6]
Indian rice grass or Indian millet seed [5]	...	..	...	1.1 [6]	3.9 [6]
Tansy mustard [5]	...	..	2.2 [6]	3.9 [6]	5.6 [6]
Narrow leaf grass [5]	...	..	...	2.8 [6]	3.9 [6]
Rocky Mountain bee plant [5]	...	..	1.4 [5]	3.5	4.6 [6]
Rocky Mountain bee plant [5]	...	..	1.6 [6]	3.4	3.9 [6]
Meats					
Broiled mutton	49.9 [4]	4.8 [4]	10.8	11.6	5.4
Large intestine	1.6	2.3 [5]	82.1	2.5	8.9
Stomach of goat	1.6	5.8	55.8 [5]	4.4	7.2
Stomach of goat	2.7 [4]	3.5 [5]	38.7	7.7	6.6
Skin of sheep	36.8	..	12.4	10.5	5.5
Blood wurst	56.2 [4]	3.3 [4,5]	40.3	5.6	6.8
Miscellaneous					
Juniper ashes [5]	...	1.7	0.0 [6]	0.2 [6]	..
Clay [5]	...	9.2	0.7	0.0	..

[1] Loss in weight of food when brought from condition as collected to air-dry condition.

[2] Percentage of moisture found in air-dry food in bringing it to an absolutely water-free condition.

[3] See explanation on pages 298 and 299.

[4] Includes benzoic acid.

[5] Average of values not agreeing within 5%.

[6] One analysis only.

[7] This sample, when air dry, contained 76% ash.

[8] Samples collected by Miss Stella Young (see p. 304).

QUANTITATIVE STUDY OF THE FOOD HABITS OF THE NAVAJO

Since the coming of the Spaniards, mutton (from either the sheep or the goat) may be called the chief food of the Navajo. On some small sections of the reservation beef supplants mutton, but this is not generally true. In contrast to the habits of the white man, the Navajo consumes all parts of the sheep or goat, including most of the alimentary canal (the different parts of the stomach and the intestines). The male reproductive organs, the brain, the soft parts of the hoofs, the ligaments of the legs, occasionally the skin after the hair has been removed, the blood and the eyes are all used. The only parts not eaten are the contents of the digestive tract, the bladder, the gall sack, the bones, the horns, and the hair. Perhaps, because the Navajo does consume practically all parts of the animal, he may have reached a dietary balance, which perfects him for life in his surroundings and which gives him a nutritional reserve resulting in his excellent physique as well as his excellent teeth (Steggerda and Hill, '36).

Very little of the corn plant is wasted. In the early summer the half-formed ears are boiled in milk and are completely eaten, even the cob. Some wheat is raised by the Navajos where they have sufficient water and some is bought from the traders or bartered from the Zuni Indians. Juniper ashes are used in place of baking powder and salt, as a flavoring for mushes and other foods and as a medicine. Clay is mixed with such foods as the wild potato and the tomatillo to counteract the unpleasant astringent effect of these foods on the mouth. Fried bread is used considerably, although it is not the main food. Baked bread is eaten freely, especially in winter. An adult man will eat three cakes (240 gm.) at one meal. The seeds of several kinds of grasses were once used for food, but very few of these seeds are used now. In times of great scarcity, any seed that could serve as a food was doubtless so used.

The quantitative study of the Navajo's food habits was not so complete and well controlled as was that made in Yucatan

for the Maya. However, the following information was secured from five reliable Navajo families concerning the amount of food eaten by each family. A family of twelve (five adults and seven children) consumes one adult goat each week or about 23 kg. of meat. The choice pieces of meat last about 3 days. During the other 4 days of the week the miscellaneous parts, intestines, feet and head, are consumed. In addition, bread, vegetables and coffee are eaten. A family of eight (three adults and five children) consumes the choice pieces of a goat in 5 days and for about 3 days thereafter the miscellaneous parts. This family also uses 11 kg. of flour every 3 days, but when much other food is available, such as vegetables, the flour lasts about 1 week. This particular family does not eat many vegetables but uses 0.5 kg. of coffee in 3 days and 2.5 kg. of white sugar each week.

Another family of ten (three adult women, two adult men and five children) consumes one goat per week, one sack (11 kg.) of wheat flour every 3 days (rarely does it last 4 days), 0.9 kg. of coffee each week, 2.5 kg. of white sugar each week and one can of condensed milk when the goats are not milking. However, from April to September this family uses from 3 to 8 liters of goat's milk per day, either as milk, butter or cheese. Another family of ten (six adults and four children) consumes one goat in 3 days. When the meat is gone, another animal is killed. For this family, 11 kg. of wheat flour last about 2 weeks. The informant added that this family uses considerable corn flour also, but little sugar. A family of fifteen, mostly adults, consumes one large mutton (25 to 30 kg.) every 2 days. In addition, corn, potatoes, bread, dried peaches and prickly pears are eaten.

In general, the average Navajo family of six or seven people consumes one goat, 10 kg. of flour, 2.5 kg. of white sugar and about 1 kg. of coffee every week, in addition to such vegetables as they may have. If available, the Navajo would make meat, chiefly mutton, 60 to 80% of his diet. The Navajos rarely keep chickens for food or eat eggs. The Navajos consume rabbits, the cottontail and the jack rabbit, and prairie dogs.

Deer and antelope used to be hunted in the mountains for food, but there are no deer now in the Navajo reservation and the antelope are protected by law. To a considerable extent the Navajos are dropping their old food habits and adopting the habits of the white man. For example, they are now turning to white flour instead of corn flour and they do not raise so many potatoes, as they buy from the trading posts. Also they buy considerable bacon in which to fry potatoes.

SUMMARY

Sixty-six samples of the commonest foods in the daily diet of the Navajo Indian of New Mexico and Arizona were analyzed with reference to their moisture, fat, nitrogen and energy content. In spite of the variability in fat and nitrogen content of the different foods, the energy content was relatively constant in most instances (between 3.5 and 4.5 calories per gram of air-dry matter). Higher energy values were found for the meats and some of the berries and seeds, which had higher percentages of fat.

The diet of the Navajo Indian consists chiefly of mutton in contrast to that of the Maya Indian of Yucatan, which is predominantly corn. Practically all parts of the animal (goat and sheep) are eaten, including most of the alimentary canal.

A study was made of the character and amount of food eaten by five Navajo families per week. The average family (six or seven persons) eats one goat each week, besides about 10 kg. of flour, 2.5 kg. of white sugar, 1 kg. of coffee and such vegetables as may be available.

ACKNOWLEDGMENTS

The majority of the Navajo foods were collected with the assistance of the late Mr. Wallace Peshlakai. Sixteen samples of food materials were kindly given to us by Miss Stella Young, head of the Home Economics department, Wingate Vocational High School, Fort Wingate, New Mexico, from her many years' collection of the seeds and fruits of native plants used by the Navajos for food. Thanks are especially

extended to Mr. and Mrs. Alexander Black of Fort Defiance, Arizona, for their thorough and conscientious checking of field notes and for critical reading of our manuscript. The determinations of the moisture content of the food samples were made by R. C. Lee and M. Stankard, the analyses for fat and nitrogen content by M. Stankard and the analyses for energy content by B. James.

LITERATURE CITED

BENEDICT, F. G. 1929 Der Oxycalorimeter. Eine Methode zur Bestimmung der Verbrennungswärme von organischen Substanzen. Abderhalden's Handb. d. biolog. Arbeitsmethoden, Abt. IV, Teil 13, S. 51.

BENEDICT, F. G., AND E. L. FOX 1925 The oxy-calorimeter. Principle and application to the determination of energy values of fuels, foods, and excretory products. Indus. and Eng. Chem., vol. 17, p. 912.

BENEDICT, F. G., AND M. STEGGERDA 1936 The food of the present-day Maya Indians of Yucatan. Carnegie Inst. Wash. Pub. no. 456, p. 155.

SHATTUCK, G. C., AND F. G. BENEDICT 1931 Further studies on the basal metabolism of Maya Indians in Yucatan. Am. J. Physiol., vol. 96, p. 518.

STEGGERDA, M., AND F. G. BENEDICT 1932 Metabolism in Yucatan: A study of the Maya Indian. Am. J. Physiol., vol. 100, p. 274.

STEGGERDA, M., AND T. J. HILL 1936 Incidence of dental caries among Maya and Navajo Indians. J. Dental Research, vol. 15, p. 233.

WILLIAMS, G. D., AND F. G. BENEDICT 1928 Basal metabolism of Mayas in Yucatan. Am. J. Physiol., vol. 85, p. 634.

THE HEAT PRODUCTION OF THE FASTING RAT IN RELATION TO THE ENVIRONMENTAL TEMPERATURE [1]

RAYMOND W. SWIFT AND RICHARD M. FORBES
Institute of Animal Nutrition, The Pennsylvania State College, State College

TWO FIGURES

(Received for publication May 8, 1939)

A study of the literature dealing with the heat production of the albino rat as affected by environmental temperature indicates that the critical temperature has not been well established, that the range in temperature over which the metabolism of this animal is constant has not been determined and that the percentage of heat eliminated by evaporation of water has not been convincingly shown to vary consistently with the environmental temperature.

Work reported by Benedict and MacLeod ('29 b) shows a constant rate of decrease in heat production as the environmental temperature is raised from 10°C. to 28°C. The graph showing the average trend of the metabolism is, unfortunately, not extended beyond 28°C. although it was considered from data not shown that, "at 28°C. and above the metabolism was essentially constant . . . ." The authors must have felt somewhat uncertain about this, however, for they also say in the same paper, "It is not impossible that temperatures higher than 28°C. may prove to lower the basal metabolism still further." No attempt was made to establish the range of temperature throughout which the metabolism remained constant.

[1] Authorized for publication on May 5, 1939, as paper no. 902 in the journal series of the Pennsylvania Agricultural Experiment Station.

Goto ('23) measured the total heat and water vapor produced by rats exposed to temperatures ranging from 5°C. to 33°C. He found the minimum heat production at 28°C. with a higher heat production prevailing above as well as below this temperature. His observations were made on four rats during the first and also during the second day of fast. Though one would expect the percentage of heat eliminated by the evaporation of water to increase steadily and consistently with rise in environmental temperature, his data, especially those of the second day of fast, show considerable lack of consistency. For instance, one rat which eliminated 20.7% of its heat by vaporization of water at an environmental temperature of 25°C. eliminated only 12.8% at 28°C. Wesson ('31) considers that the basal metabolism of rats should be determined at 28°C. Schwabe and associates ('38) consider the range of thermal neutrality to be 28°C. to 30°C. Males and Magaz ('29) found the metabolism of fasting rats to be constant from 30 to 33°C. and only slightly variable throughout a range in temperature from 29 to 33.5°C. Prior to the measurement of metabolism the rats were kept at a temperature of 8 to 10°C., feed being withdrawn 10 to 14 hours before the experiment began. Two preliminary periods of ½ hour each followed; during the first one the rats were kept at an environmental temperature of 13 to 15°C. and during the second, at the temperature of the experiment.

Terroine and Trautmann ('27) working with many species of animals found that the minimum heat production with rats occurred at an environmental temperature of 33°C. Only two rats were used to secure a very limited number of observations at a given temperature.

By the use of animals which were similar as to age, weight and sex, and in the postabsorptive state, the primary purpose of this study was to determine 1) the critical temperature for the albino rat, 2) the range in environmental temperature over which the metabolism remains constant, and 3) the percentage of heat eliminated by the evaporation of water. In order to accomplish these objectives, it was considered neces-

sary to accumulate enough reliable data thoroughly to establish each individual point of observation with a reasonably small probable error, in order that the significance of the differences in findings at different temperatures could be statistically evaluated. It was felt that the short periods of observation ordinarily used at many laboratories are quite unsatisfactory, on account of the possibility of technical errors as well as the unavoidable physiological lag of the animal in response to a change in environmental temperature.

The open circuit Haldane method of procedure was followed, the oxygen absorption during 8 hours being weighed as the sum of the weights of CO_2 and water vapor eliminated minus the loss in weight of the rat and container. The CO_2 and water were weighed at intervals of 2 hours, the measurement for the first period of 2 hours being excluded. Thus, the respiratory quotient and the total oxygen consumption were derived from 8-hour periods, while the hourly heat and water vapor were derived from the carbon dioxide and water vapor of the last 6 hours.

The water produced was absorbed in glass-stoppered U-tubes containing pumice saturated with H_2SO_4, and the CO_2 was absorbed by flake NaOH mixed with pumice in similar tubes as described by Forbes ('39). These tubes were weighed on the ordinary chemical balance.

The food was withdrawn 15 hours before the start of the experiment, or 17 hours before the beginning of the 6-hour period of measurement of heat production and water vapor.

The rat was put into a screw-cap bottle of about 4 liters capacity, and after weighing to the nearest milligram on a chainomatic balance, the bottle containing the rat was immersed in a water bath, the surface of the water coming to within about 1 inch of the top of the bottle. Two lead weights attached to the bottle while in a water bath caused it to rest securely on a false bottom of hardware cloth.

Two brass tubes soldered to the metallic bottle caps served as inlet and outlet for the air current. The inlet tube went nearly to the floor of the bottle while the outlet extended only

an inch below the cap. Dry CO_2-free air was supplied at the rate of 2 liters per minute, this rate of flow being measured by means of a previously calibrated air-speed indicator.

A short thermometer fastened to the longer brass tube was used to indicate the temperature of the air within the bottle. The heat from the animal raises the air in the bottle to a temperature somewhat above that of the surrounding water bath. For instance, when the bath temperature was 4.0°C. the bottle temperature was 7.5°C.; when the bath temperature was 24.0°C. the bottle thermometer read 25.0°C. The difference between the temperature of the bottle and bath was 0.5°C. at a bath temperature of 27.5°C., and became progressively smaller at higher temperatures. The thermometer was read after raising the bottle out of the bath (permitted by flexible rubber tubing connections), and while holding the bottle at such a height that the top of the mercury in the thermometer was level with the eye. If the animal was resting against the bulb of the thermometer or was very close to it, the reading was improperly high and was not recorded. Otherwise the bottle temperature was as constant as that of the bath. The temperature of the air in the bottle was considered to be the environmental temperature to which the rat was exposed.

The rat rested upon a floor of ¼ inch mesh hardware cloth supported about 1½ inches above the bottom of the bottle. The bottom of the bottle was covered about ½ inch deep with paraffin oil. As shown by trial, the amounts of water and CO_2 coming from the excreta covered with the oil were entirely negligible. Water evaporated from the urine clinging to the screen floor would give results too high for the percentage of heat eliminated by the evaporation of water, but would cause no error in the determination of the total heat production. However, in this connection it was observed in the few cases when no urine or feces were voided, that the results were indistinguishable from those when urine was present. Though urine was voided in most of the 8-hour periods, at all tempera-

tures, feces were rarely found after the periods conducted at 28°C., 30°C. or 31°C.

The water bath, the chamber and the use of oil to prevent evaporation of water from the excreta were devised by E. B. Forbes, and the bath, with accessory equipment, was constructed by Precision Scientific Co.

The bath was connected with an insulated tank containing ice water during experiments conducted below room temperature and hot water during experiments conducted above room temperature (fig. 1). A thermostat and solenoid valve operated to take water from the reservoir of cold (or hot) water

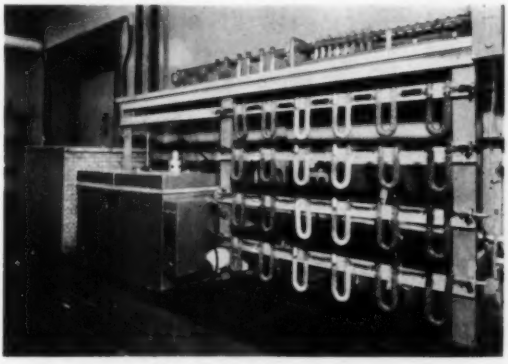


Fig. 1 Constant temperature bath and accessory equipment.

when needed to maintain the temperature of the bath constant to within ± 0.4°C. or less. A pump circulated the water within the bath for the equalization of its temperature. The bath contained two bottles, and the metabolism of two rats was determined simultaneously.

After removal from the bath the bottle was wiped dry before weighing. When the bath was used at the lower temperatures the bottle, after removal, was brought to room temperature with the aid of warm water to facilitate drying and to prevent subsequent condensation of moisture on the surface.

The tightness of the bottle and absorption tubes was determined by frequent tests with a mercury manometer, and in addition several 8-hour blank determinations which included weighing the bottle and U-tubes as in an ordinary experiment were made during a wide range of temperatures. All blank tests were negligible.

The heat and water vapor produced were determined at each temperature by several 8-hour observations as shown in table 1. The chronological order of the periods of observation is also given in the table.

A total of seven male rats were used in obtaining the data though only six rats were used throughout the entire series. Thus, the fourteen 8-hour observations at 31°C. involved the use of four rats during 2 days each, and two rats during 3 days each.

The heat production of all rats was calculated to a live weight of 325 gm., this being the actual live weight of the rats at the start of the experiment. The calculation was made in accord with the two-thirds power of the live weight. The average live weight at the end of the experiment was 341 gm. No large correction of the heat production was nceessary, therefore, in any one of the series of observations.

The live weight of the rats was taken at the start of the period of metabolism measurement, 15 hours after the withdrawal of feed. The rats were 21 weeks old at the start of the experiment and 38 weeks old at the end. The respiratory quotients were not corrected for the protein metabolism.

DISCUSSION OF RESULTS

No attempt was made to measure the activity of the rats. They were visible at all times through a glass window in the cover of the bath and were remarkably quiet at all temperatures. Schwabe and associates ('38) have shown that activity is without effect on the respiratory quotient.

The most significant measure available regarding the variability of results is presented in table 1. The average of the coefficients of variability of the heat production is 4.52%, the

average probable error of the nine series being ±0.016 Cals. It is evident, therefore, from the concordant results at a given temperature, that the activity was relatively constant and that this factor contributed but little to the variability of the results.

The correct values for the heat production at all temperatures may be somewhat higher than as observed during rest, though the long periods of observation in the present experiment seem to compensate for the lack of a record of activity. We therefore agree with Benedict and MacLeod ('29 a) that, "in practically all cases the graphic record of activity can be dispensed with, especially if it is possible to observe the animal continually." It was observed that at 33°C. and at 35°C. the rats were not only quiet but were sprawled out in an apparent attempt to expose as much surface as possible to the air. After exposure to a temperature of 35°C. for 8 hours the rats appeared to be as wet as though dipped in water.

It has been pointed out by several investigators that the height of the heat production may be affected by the temperature to which the animals have been exposed prior to the time of actual heat measurement. Schwabe and associates ('38) found that exposure to low environmental temperatures (7.8°C. to 12.2°C.) for a period of 60 days prior to the measurement of the fasting metabolism at 29°C. resulted in an increase of about 12% in heat production. In the work reported by Males and Margaz ('29) the very low temperatures to which the rats were exposed prior to the metabolism measurements may have affected their results. The temperature to which the rats used in the present study were exposed when not on experiment and while receiving feed was about 25°C., which presumably did not affect the results obtained. This temperature (25°C.) corresponds closely to that prevailing in other laboratories and seems, therefore, to represent conditions ordinarily encountered. The rats were kept in individual cages in a room containing a rat colony which

has been in a strong, healthy condition during a period of 10 years.

The small though consistent rise in respiratory quotient with rise in environmental temperature indicates that the temperature exerts a qualitative as well as a quantitative effect on the metabolism. Short exposures to cold with human subjects accompanied by shivering have no effect on the respiratory quotient (Swift, '32). However, Kayser and Dell ('37) and Kayser ('37) working with rats, guinea pigs and pigeons have shown that the increase in metabolism due to exposure to cold which occurs before the point of shivering is reached is entirely due to the oxidation of fat, even when an abundance of carbohydrate is available. In this experiment shivering did not occur and the contention of the above authors is apparently verified in view of the fact that a statistical study shows that the odds are millions to 1 that the respiratory quotient at 30°C. is greater than at 25°C. Though the computation of the respiratory quotient of the 'extra metabolism' involves unavoidable magnification of error, it seems significant that this value for the low temperatures is 0.68 whereas for the temperature 35°C. it is 0.85.

It may be remarked, in passing, that the use of a lower caloric value for CO_2 with rise in respiratory quotient exerts only a negligible effect on the computed heat production. Respiratory quotients similar to those obtained in this work at 28 to 30°C. (about 0.753) have been reported by various other workers. Wesson ('31) found a respiratory quotient of 0.769 with rats fasted 24 hours, while Schwabe and associates ('38) report an average value of 0.755 representing some 200 animals.

The data in table 1 and the graphic presentation in figure 2 indicate with apparent certainty that the critical temperature of the fasting albino rat is approximately 30°C. There was the possibility that the repeated fasts produced a lowered basal metabolism, even though the total length of each fast was only 23 hours, and such fasts occurred on an average only about twice a week. As shown in table 1 the series of four-

teen observations at 31°C. directly followed the series at 28°C., so there was very little likelihood that the lesser heat production at 31°C., as compared to that at 28°C., was due to an accumulative effect of underfeeding.

However, since 28°C. has been generally accepted as the critical temperature, special effort was made to establish this particular point beyond possibility of doubt. In order then to prevent misinterpretation due to possible influence of

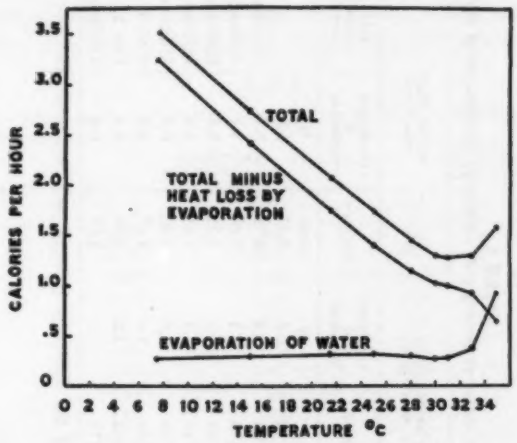


Fig. 2 The character of the heat disposal of the fasting albino rat at environmental temperatures ranging from 7.5° to 35°C.

previous fasting on the value obtained as the critical temperature and to verify the finding that the minimum heat production does not occur at 28°C., eight colony rats, never before fasted, were used in determining the metabolism at 31°C., a point within the range of thermal neutrality and, subsequently, at 28°C. Thus the order of experimentation of the first series was reversed to avoid the possibility of a lowered metabolism at 31°C. due to previous fasting. The average live weight of these rats was 297 gm. The hourly heat production was computed to a live weight basis of 325 gm., as were the corresponding values in table 1. The individual data

TABLE 1

Total heat produced and water vaporized with change in environmental temperature

ORDER OF EXPERIMENTS	NUMBER OF 8-HOUR OBSERVATIONS	TEMPERATURE	RESPIRATORY QUOTIENT		TOTAL HEAT PER HOUR			HEAT ELIMINATED BY VAPORIZATION OF WATER	
			Average	Coefficient of variation	Average	Coefficient of variation	Odds of significance[1]	Per cent of total	Coefficient of variation
		°C.		%	Cal.	%		%	%
1	8	7.5	0.710	0.8	3.51	2.8		7.9	4.9
2	11	15.0	0.723	0.8	2.72	3.7	Billions to 1	10.9	8.1
3	15	21.5	0.729	1.1	2.08	3.1	Billions to 1	15.5	8.0
4	13	25.0	0.740	1.3	1.72	3.9	Billions to 1	18.7	8.0
5	15	28.0	0.747	1.3	1.45	4.6	Billions to 1	20.5	9.2
8	11	30.0	0.759	1.0	1.30	6.2	Millions to 1	20.8	4.2
6	14	31.0	0.759	1.4	1.27	4.8	2 to 1	22.2	6.0
7	13	33.0	0.763	1.5	1.29	5.1	1 to 1	27.8	10.0
9	6	35.0	0.775	1.1	1.57	6.6	1 billion to 1	59.2	13.1

[1] These odds represent the significance of the difference between each heat production value and the one at the preceding lower temperature.

are given in table 2. The evidence is again overwhelming that fasting rats have a higher heat production at 28°C. than at 31°C.

These rats were 15 weeks of age while those of the first series (table 1) were about 32 weeks old when used at these temperatures. Though this experiment was not designed to show the effect of age on basal metabolism, the higher level of heat production of the younger rats at both temperatures indicates that metabolism decreases with age—in contrast to the observations of Benedict and MacLeod ('29). However, our results are concerned with two groups of rats at ages of

TABLE 2

Heat production at an environmental temperature of 31°C. compared with 28°C.

RAT NUMBER	HEAT PRODUCTION PER HOUR		HEAT LOST BY EVAPORATION OF WATER	
	28°C.	31°C.	28°C.	31°C.
	Cal.	*Cal.*	%	%
67	1.48	1.41	18.7	24.4
68	1.52	1.41	16.6	19.8
69	1.56	1.44	18.8	22.7
70	1.71	1.49	23.6	25.1
71	1.49	1.36	18.7	23.5
72	1.67	1.47	18.8	26.3
73	1.58	1.48	19.5	22.4
74	1.63	1.45	23.2	26.8
Av.	1.58	1.44	19.7	23.9

about 3 and 7½ months of age. Black ('29) has reported a higher basal metabolism in very old rats (700 days) than in younger ones (7 to 8 months).

It may be observed from table 1 that although the percentage of heat eliminated by vaporization of water increases steadily with rise in environmental temperature, the amount of heat thus eliminated is essentially constant over an extended range of temperatures and does not increase materially until shortly before the point of hyperthermal rise is reached.

SUMMARY

The heat production of the albino rat in the postabsorptive state was determined in 122 8-hour periods of measurement

at environmental temperatures ranging from 7.5° to 35.0°C. The critical temperature for the fasting rat was found to be 30°C. The metabolism was constant over a range of temperatures extending from 30°C. to 33°C. The percentage of the total heat eliminated as latent heat of water vapor increased steadily from 7.9% to 59.2%, with rise in environmental temperature from 7.5°C. to 35°C., though the absolute amount of heat eliminated in this manner was practically constant up to 31°C., above which a definite increase took place.

LITERATURE CITED

BENEDICT, F. G., AND GRACE MACLEOD 1929 a The heat production of the albino rat. I. Technic, activity control and the influence of fasting. J. Nutrition, vol. 1, p. 343.

———— 1929 b The heat production of the albino rat. II. The influence of environmental temperature, age and sex; comparison with the basal metabolism of man. J. Nutrition, vol. 1, p. 367.

BLACK, A. 1939 The effect of protein and exercise at different ages on the basal metabolism. J. Nutrition, vol. 17, p. 361.

FORBES, E. B. 1939 Satisfactory substitute for soda lime in respiration calorimetry. Science, vol. 89, p. 252.

GOTO, KIKO 1923 Beitrag zur Kenntnis der chemischen Wärmeregulation der Säugetiere. III. Wärmeregulation der wiessen Ratte. Biochem. Zeitschr., Bd. 135, S. 107.

KAYSER, C. 1937 Variations du quotient respiratoire en fonction de la température du milieu chez le rat, le pigeon, et le cobaye. Compt. rend. soc. biol., vol. 126, p. 1219.

KAYSER, C., AND P. DELL 1937 Signification des variations du quotient respiratoire en fonction de la température du milieu chez le hamster réveillé. Compt. rend. soc. biol., vol. 126, p. 698.

MALES, BRANIMIRO, AND JAIME MAGAZ 1929 Su una zona die neutralità termica nel metabolismo basale del ratto albino. Boll. Soc. Ital. Biol. Sper., vol. 4, p. 461.

SCHWABE, E. L., F. E. EMERY AND F. R. GRIFFITH, JR. 1938 The effect of prolonged exposure to low temperature on the basal metabolism of the rat. J. Nutrition, vol. 15, p. 199.

SWIFT, R. W. 1932 The effects of low environmental temperature upon metabolism. I. Technic and respiratory quotient. J. Nutrition, vol. 5, p. 213.

TERROINE, É. F., AND S. TRAUTMANN 1927 Influence de la température extérieure sur la grandeur des échanges chez les homéothermes et loi des surfaces. Ann. Physiol. et Physico-Chim. Biol., III, pp. 422–457.

WESSON, L. G. 1931 The metabolic rate and respiratory quotients of rats following the ingestion of dextrin and during fasting. J. Nutrition, vol. 3, p. 503.